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"We are first taught to think inside the box. Then we are taught to think outside the box. The main question though is… Who put the box there?!"

Unknown author

To my parents

To Gabi, for being my strength,

my inspiration and my life

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Abstract

There is substantial evidence that exercise has anabolic effects on bone tissue. Nevertheless, exercise induced bone gains are more evident at young ages, and seem to be negligible in the elder and in post-menopausal women. However, most studies regarding the effectiveness of exercise in the prevention of bone fragility have used just bone mineral density (BMD) increases as the main end-point for assessing the intervention effectiveness despite that bone strength depends on a cluster of properties and not just on BMD. Therefore, the effectiveness of exercise as a strategy to prevent bone strength losses with age or estrogen deficiency may have been largely underappreciated by studies in which effectiveness was merely monitored by analyzing changes in BMD. Consequently, the main objective of this work was to investigate how ageing and/or estrogen deficiency affect several of the bone material and geometrical properties (trabecular microarchitecture, cellular density, radiographic absorptiometry, mineralization degree, bone geometry) that determine its mechanical resistance (strength and stiffness) as well as to determine the possible role of physical activity in the prevention of those changes. To accomplish these objectives we used male *C57BL/6* mice that were subjected through life (from 2 to 25 months of age) to sedentary or physically active housing conditions. This model enabled us to determine the extent to which sedentary behavior contributed to the bone changes associated with ageing. In another set of experiments we have used female *Wistar* rats that were first ovariectomized (or sham-operated) at 5-months of age, and afterwards housed until 14-months of age in sedentary or physically active conditions. This model allowed us to appreciate how voluntary physical activity was affected by estrogen levels as well as to determine the role of regular exercise in the prevention of estrogen-loss induced bone changes.

Our overall results show that in the elder male mice, age related bone losses were significantly greater in the sedentary animals than in the physically active animals, suggesting therefore that physical inactivity significantly enhances bone losses associated with age. Our results also demonstrate that estrogen exerted a pivotal role in the female rat motor activity pattern since low estrogen levels induced a significant

decrease in physical activity levels and were thereby a main contributor to estrogen loss associated sarcopenia. Regular physical activity however, was shown to significantly prevent the decline in bone tissue quality that occurs with age and estrogen loss. The overall conclusion of the present work is that regular physical activity is able to prevent at some extent the bone tissue quality declines associated with ageing and estrogen loss, contributing thereby to the prevention of bone fragility associated with these conditions.

Resumo

Existem bastantes evidências de que o exercício físico tem efeitos anabólicos sobre o tecido ósseo. Porém, os ganhos de massa óssea associados ao exercício parecem ocorrer essencialmente em idades jovens, parecendo ser negligenciáveis em idosos e mulheres pós-menopausicas. Contudo, a maioria dos estudos que analisam a eficácia de programas de exercício na prevenção da fragilidade óssea, utilizam como medida principal de eficácia a determinação de alterações na densidade mineral óssea (DMO). Porém, a capacidade de resistência do tecido ósseo depende de um conjunto alargado de propriedades e não apenas da DMO. Desta forma, os reais efeitos benéficos do exercício na prevenção da fragilidade óssea associada à idade e à perda de estrogénio poderão ter sido subvalorizados por estudos prévios que se basearam apenas em alterações da DMO. Consequentemente, este trabalho teve como principal objectivo investigar de que forma a idade e/ou a ausência de estrogénio afectam um conjunto alargado de propriedades materiais e geométricas do fémur (microarquitetura trabecular, densidade celular, geometria, densidade radiográfica, concentrações de cálcio e fosfato) determinantes para a sua resistência mecânica (força máxima e rigidez óssea), assim como investigar os possíveis efeitos do exercício físico na prevenção desse conjunto de alterações. Para atingir estes objectivos, foram utilizados murganhos machos da estripe *C57BL/6* submetidos durante toda a vida (desde os 2 até aos 25 meses de idade) a condições de sedentarismo ou de actividade física regular. Este modelo permitiu avaliar de que forma é que o sedentarismo contribuiu para o agravamento das alterações ósseas associadas ao envelhecimento. Num outro conjunto de experiências utilizaram-se ratos fêmea da estirpe *Wistar* que foram ooforectomizados (ou operados sem remoção dos ovários) aos 5 meses de idade e seguidamente alojados até aos 14 meses de idade em condições de sedentarismo ou de actividade física regular. A utilização deste modelo permitiu analisar de que forma é que a actividade motora dos animais é influenciada pelos níveis de estrogénio assim como determinar a influência da prática de exercício físico regular na prevenção das alterações ósseas associadas à perda de estrogénio.

Os resultados demonstraram que, nos ratinhos machos idosos, as alterações do tecido ósseo associadas ao envelhecimento foram significativamente mais extensas nos animais sedentários comparativamente aos animais fisicamente activos, o que sugere portanto que o sedentarismo exacerba significativamente o envelhecimento do tecido ósseo. Os resultados demonstraram igualmente que o estrogénio exerce um papel fundamental na regulação da actividade motora dos ratos fêmea já que a redução dos níveis de estrogénio induziu uma diminuição acentuada da actividade física voluntária destes animais. Este facto teve uma influência determinante na diminuição da massa muscular verificada após a ooforectomia. A realização de actividade física regular porém, demonstrou ser uma estratégia eficaz na prevenção da deterioração da qualidade do tecido ósseo associada à idade e à perda de estrogénio. A conclusão geral deste trabalho é que a actividade física regular é capaz de prevenir, em certa medida, a deterioração das propriedades do tecido ósseo resultantes do envelhecimento e da perda de estrogénio, contribuindo dessa forma para a prevenção da fragilidade óssea associada a estas condições.

Resumé

Il y a plusieurs évidences que l'exercice physique a des effets anaboliques sur le tissu osseux. Néanmoins, les profits de la masse osseuse associés à l'exercice semblent se produire essentiellement pendant le jeune âge, semblant être négligeables dans les personnes âgées et les femmes post-ménopausiques. Malgré cela, la majorité des études qui analysent l'efficacité des programmes d'exercice dans la prévention de la fragilité osseuse, utilisent comme mesure principale d'efficacité la détermination des modifications dans la densité minérale osseuse (DMO). Cependant, la capacité de résistance du tissu osseux dépend d'un ensemble élargi de propriétés et non seulement de la DMO. Ainsi, les réels effets bénéfiques de l'exercice dans la prévention de la fragilité osseuse associé à l'âge et à la perte d'estrogène peuvent avoir été sous-valorisés par des études préalables qui ne se sont basées que sur les modifications de la DMO. En conséquence, ce travail a eu comme principal objectif faire des recherches sur la façon dont l'âge et/ou l'absence d'estrogène affectent un ensemble élargi de propriétés matérielles et géométriques du fémur (microarchitecture trabeculaire, densité cellulaire, géométrie, densité radiographique, concentrations de calcium et phosphate) déterminantes pour sa résistance mécanique (force maximum et rigidité osseuse), ainsi que faire des recherches sur les possibles effets de l'exercice physique dans la prévention de cet ensemble de modifications. Affin d'atteindre ces objectifs, nous avons utilisé des souris mâles d'extirpe *C57BL/6* soumises pendant toute leur vie (depuis l'âge 2 jusqu'à 25 mois) à des conditions de soit de sédentarité soit d'activité physique régulière. Ce modèle a permis d'évaluer de qu'elle façon la sédentarité a contribué à l'aggravation des modifications osseuses associés au vieillissement. Dans un autre ensemble d'expériences nous avons employé des rats femelles de la lignée *Wistar* qui ont été ooforectomisées (ou opérés sans déplacement des ovaires) à l'âge e 5 mois et ensuite logés jusqu'à l'âge de 14 mois dans des conditions de sédentarité ou d'activité physique régulière. L'utilisation de ce modèle a permis d'analyser de qu'elle façon l'activité motrice des animaux est influencée par les niveaux d'estrogène ainsi que déterminer l'influence de la pratique de l'exercice physique régulier dans la prévention des modifications osseuses associés à la perte d'estrogène.

Les résultats ont démontré que, dans les petites souris mâles âgées, les modifications du tissu osseux associés au vieillissement ont été significativement plus étendues dans les animaux sédentaires comparativement aux animaux physiquement actifs, ce qui suggère donc que la sédentarité exacerbe significativement le vieillissement du tissu osseux. Les résultats ont démontré également que l'estrogène exerce un rôle fondamental dans le règlement de l'activité motrice des souris femelles puisque la réduction des niveaux d'estrogène a induit une diminution accentuée de l'activité physique volontaire de ces animaux. Ce fait a eu une influence déterminante dans la diminution de la masse musculaire vérifiée après l'ooforectomie. La réalisation d'une activité physique régulière néanmoins, a démontré être une stratégie efficace dans la prévention de la détérioration de la qualité du tissu osseux associé à l'âge et à la perte d'estrogène. La conclusion générale de ce travail est que l'activité physique régulière est capable d'empêcher, dans une certain mesure, la détérioration des propriétés du tissu osseux résultant du vieillissement et de la perte d'estrogène, en contribuant ainsi à la prévention de la fragilité osseuse associé à ces conditions.

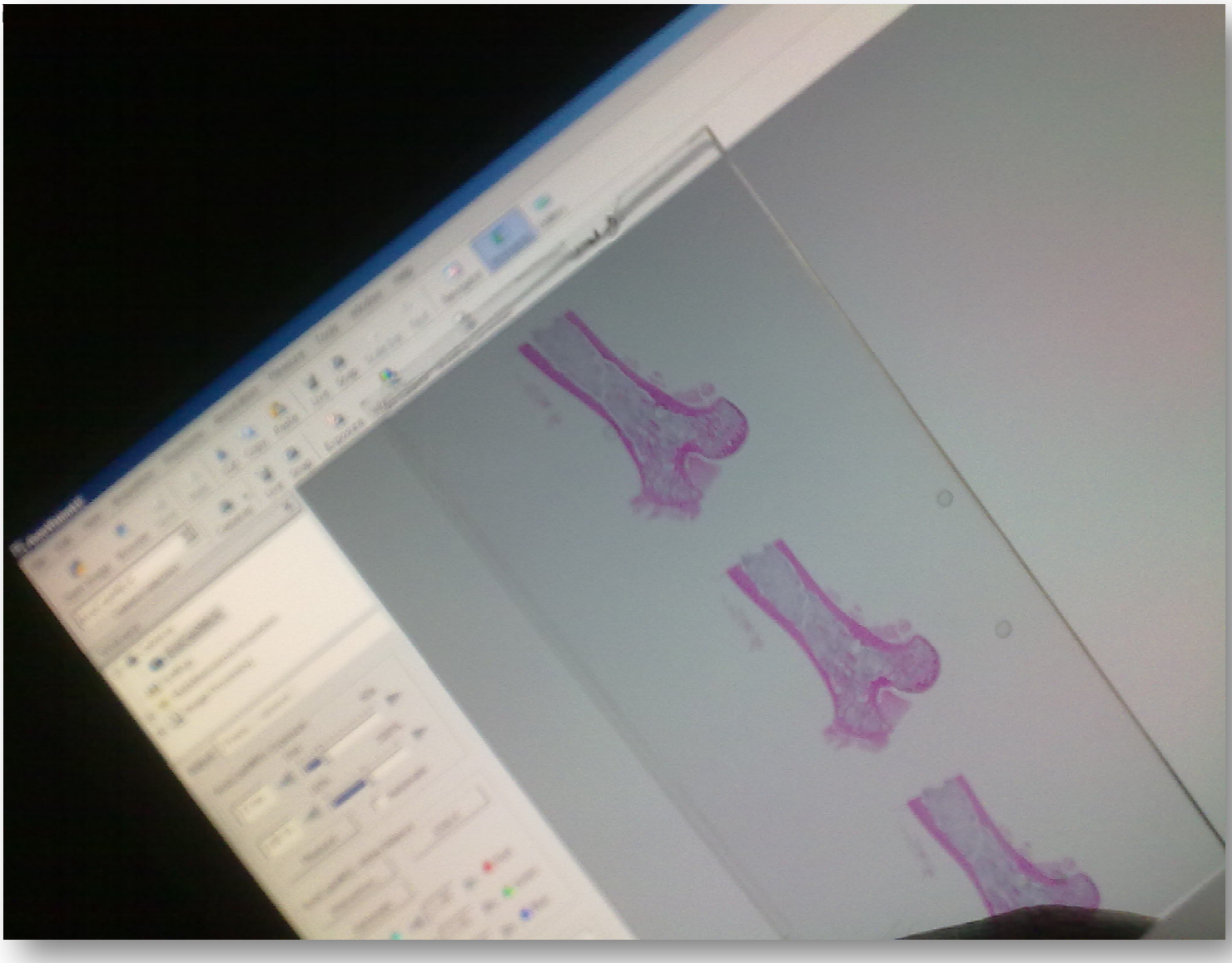
List of abbreviations

AGEs	Advanced glycation end products
ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
B.Dm	Bone diameter
BMC	Bone mineral content
BMD	Bone mineral density
BV/TV	Trabecular bone volume fraction
$[Ca^{2+}]_i$	Intracellular calcium concentration
CFMA	Compression force moment arm
COX-2	Cyclooxygenase-2
Ct.Th	Cortical bone thikness
Cx43	Connexin43
DXA	Dual-energy X-ray absorptiometry
ECM	Extracellular matrix
ER	Estrogen receptors
ERK	Extracellular signal-regulated kinases
FNAL	Femoral neck axis length
FNW	Femoral neck width
GM-CSF	Granulocyte-macrophage colony-stimulating
GPCR	G-protein coupled receptors
GSK-3β	Glycogen synthase kinase-3 beta
HRT	Hormone replacement therapy
IL	Interleukin
INF-γ	Interferon-gamma
Ma.Dm	Marrow diameter
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MHC	Myosin heavy chain
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
NFAT2	Nuclear factor of activated T-cells-2
•NO	Nitric oxide
NSA	Femoral neck-shaft angle
OPG	Osteoprotegerin
OVX	Ovariectomy/ovariectomized
PGE2	Prostaglandin E2

PKA	Protein kinase A
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor kappa-B
RANK-L	Receptor activator of nuclear factor kappa-B ligand
ROS	Reactive oxygen species
SHAM	Sham-operated
Tb.N	Trabecular number
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor-alpha
WHO	World health organization
Wnt/β-catenin	Wingless-int/beta-catenin
µCT	Microcomputed tomography
µMRI	Magnetic resonance microimaging

CHAPTER I

Introduction



During the last century, the implementation of several public health measures as well as the general increase in the quality and availability of medical care, have contributed to the overall enhancement of life expectancy, making the elderly the fastest growing age group in industrialized countries [402]. The reduction of the mortality in young and adults has led to that people now are living long enough to experience more frequently degenerative disorders which became the major causes of morbidity and mortality in industrialized countries [87].

Osteoporosis is one of the most common age-related diseases [118], affecting a significant portion of the elder population and of post-menopausal women [201]. It is defined conceptually as low bone mass and microarchitectural deterioration of bone tissue [408] while in the clinical practice osteoporotic patients are operationally defined as those having a bone mineral density (BMD) that is 2.5 or more standard deviations lower than the average bone mass of healthy young adults [408]. Its clinical significance arises from the increased risk of fracture following minimal trauma, which has substantial consequences both to the individual as well as to the society [127]. Although osteoporotic fractures may occur at multiple skeletal sites, the most commonly affected are the femoral neck, vertebral bodies and distal radius. Epidemiological studies [569] estimate that about half the women and one-fifth of man will experience a fragility fracture after 50 years of age, and that even if the current incidence rate remains stable, absolute numbers of fractures should rise as the average age of the population increases [504, 260]. In addition, the occurrence of a fragility fracture significantly increases the risk of having subsequent fractures [469, 287, 370]. Osteoporotic fractures are a considerable cause of disability to the individual [128, 369] and frequently lead to premature death [92, 58, 246]. For instance, during the first year following femoral neck fracture, there is an increase of more than 2-fold and of more than 3-fold in the mortality risk in women and men, respectively [97]. Osteoporotic fractures are also a huge economic burden to the society due to health care costs associated with medical treatments [452] and losses of productivity [433, 344], and it is estimated that the annual costs should rise substantially in the course of the following years [84].

The increased bone fragility in osteoporosis is a consequence of the overall loss of bone tissue, microarchitectural deterioration [100], and alterations in extracellular bone matrix properties [483], which are to a great extent the outcome of imbalances in bone turnover [185]. Bone tissue is continuously being remodeled throughout life by the action of osteoclasts and osteoblasts that coordinately perform bone resorption and bone formation, respectively, allowing thereby the renewal of damaged bone tissue [461] as well as the buffering of blood calcium levels. It is the unbalance in this process that is in the origin of osteoporosis and on most cases of bone fragility.

According to the estrogen-centric model of osteoporosis [456], bone losses take place as a result of estrogen deficiency. Estrogen exerts a pivotal role on the activity of bone cells, namely by inhibiting the differentiation and promoting the death by apoptosis of osteoclasts [76] as well as by inhibiting the differentiation of early osteoblast progenitors [139], thereby slowing the rate of bone turnover. Consequently, the rapid decrease in estrogen concentration that follows menopause in women, leads to an increase in bone turnover rate [185] with resorption exceeding bone formation. This results in an initial period of rapid bone loss, especially trabecular bone [392], followed by a more slower period of bone loss [456] which is also similar to what is observed in men. The more progressive loss of bone tissue that happens in men as well as in elder women is considered to be mostly the consequence of secondary hyperparatiroidism [366] due to effects that low estrogen concentration has on extraskeletal tissues involved in the regulation of calcium homeostasis, namely the kidney [365] and the intestine [188]. Only more recently, the role of oxidative stress and of cell senescence in the pathogenesis of both post-menopausal and age related osteoporosis has began to assume increasingly importance [339, 280, 343].

Despite the relevance of low bone mass for bone fragility [345], other abnormalities also contribute to fracture risk, most importantly the loss of skeletal muscle mass [538] that occurs with age [144] and at the onset of menopause [143] and that is designated as sarcopenia. Besides effecting directly bone cells and other tissues involved in the regulation of calcium homeostasis, there is compelling evidence suggesting that low estrogen concentration also promotes skeletal muscle wasting

[143] which may increase substantially the fracture risk namely due to the higher probability of falling [538]. For instance, decreases in skeletal muscle mass and strength are a well documented outcome of menopause [143] and they are shown to be prevented by estrogen replacement therapy [520, 468]. Low estrogen levels are also shown to have a negative impact on skeletal muscle energy metabolism since the mitochondria are estrogen sensitive organelle [101] and estrogen administration to ovariectomized (OVX) animals enhances respiratory chain activity [429]. Quite interestingly, there is also compelling evidence suggesting that estrogen regulates motor activity [196]. For instance OVX rats have been reported to run significantly less when compared to age matched intact controls [552] and estrogen replacement effectively reverses OVX-induced physical inactivity in mice in a dose-dependent fashion [131, 196]. It is therefore possible that menopause-induced sarcopenia might not just be caused by the effects of low estrogen on skeletal muscle, but it may also be consequence of skeletal muscle disuse due to decreases in voluntary physical activity.

The progressive loss of skeletal muscle mass is also observed in the ageing male [144], and despite that in the male there is no evidence of an abrupt decrease in physical activity as there is in the female following estrogen loss, there is substantial evidence showing that males also experience a significant decrease in voluntary physical activity levels throughout life [233]. Therefore, it is possible that at the resemblance of females, age-related sarcopenia might be associated not just with the ageing process *per se*, but also with progressively lower physical activity levels. Most importantly, and considering the importance that regular exercise has for the maintenance of bone heath [176, 292, 78], it is also possible that the increased bone fragility observed in the ageing male might not just result from imbalances in calcium homeostasis or cell senescence due to the ageing process *per se*, but may also be a significant outcome of the decline in physical activity levels over the years.

One of the major challenges raised by osteoporosis is that it remains clinically silent until it manifests itself through the occurrence of a fragility fracture. This makes therefore essential the establishment of adequate screening strategies that allow reliable, timely and cost-effective detection of those at risk of fracture. Currently, dual-

energy X-ray absorptiometry (DXA) derived measures of BMD are the gold-standard for diagnosing osteoporosis and for the clinical assessment of fracture risk. However and regardless of the epidemiological evidence showing that BMD values correlate with fracture risk [345] this variable is only able to predict a portion of the individuals fracture risk [500], which leads to insufficient recognition of those that are more likely to experience a fracture [423, 582]. For instance, there are reports showing that 66% and 79% of non-vertebral fractures occur in women and men without diagnosed osteoporosis, respectively [496]. Additionally, age related decreases in femur strength are estimated to be steeper than decreases in BMD [270], suggesting that the population at risk for fragility fractures is substantially underestimated according to the DXA diagnosis criteria, which has led to the discouragement of osteoporosis screening programs relying merely in BMD as an endpoint [345]. Despite the clinical relevance of BMD and of low bone mass as a component of fracture risk, it is now well established that bone strength is influenced by a set of orchestrated features [239] and that skeletal fragility is the final outcome of the interaction between several material and structural bone properties [360]. Hence, the use of BMD alone for estimating fracture risk is largely insufficient and likely misleading as it provides limited information about bone tissue material composition and structural design [360]. In order to improve the assessment of bone fragility, several investigations have been performed with the objective of determining how other than BMD bone properties correlate with bone strength, such as bone geometry [82], trabecular microarchitecture [470] or bone turnover [184]. However, the existence of conflicting results [15, 48] has difficult the assessment of the contribution that each of these properties have for bone resistance.

During the last decades, several pharmacological agents have become available for treatment of osteoporosis and their effectiveness is well demonstrated by large placebo controlled trials [135, 56]. Bisphosphonates are among the most commonly used. Nevertheless, as any drug, these treatments have adverse secondary effects, namely gastrointestinal disorders [160], osteonecrosis of the jaw [55, 356] and increased occurrence of atypical femoral fractures [531, 505]. Recently a significant

increase in the incidence of atrial fibrillation was also reported as a serious adverse effect in patients receiving some forms of bisphosphonates [57, 427, 4]. Likewise, poor compliance [430, 79, 60, 359] and persistence [114, 431] to the treatments are serious shortcomings in patients receiving drugs against osteoporosis. Prevention of fractures with pharmacological treatments is also costly [128], and the treatment cost-effectiveness is a significant matter of concern [169, 251] as health care resources are inevitable limited and the population at risk for osteoporotic fractures is significant and is increasing [84]. The cornerstone in osteoporosis treatment should therefore not just be treating those with established bone loss, but specially preventing skeletal fragility through non-pharmacological measures in order to reduce or delay the requirement of pharmacological treatments. Several non-pharmacological measures, like a calcium and vitamin-D rich nutrition [546] and exercise [255, 159] have the potential of reducing the risk of developing osteoporosis.

There is extensive evidence showing that exercise is beneficial to the skeleton [222, 219, 220, 205, 397, 579, 576, 603] and that disuse seriously jeopardizes bone structure [304, 275, 299]. For instance, observational studies show that bone mass is higher in athletes compared to sedentary controls [219, 220, 603] and that BMD is higher in the dominant, compared to the non-dominant, arm in tennis players [294, 206, 205, 358]. Conversely, microgravity [304, 275] and prolonged bed rest [299] are major skeletal stimulus that lead to rapid bone losses. Several studies however suggest that exercise-induced increases in BMD occur especially during growth [332, 276] which would, by increasing peak bone mass [214, 216], grant extra protection against bone losses later in life, thereby preventing osteoporosis. Nevertheless, there is compelling evidence that exercise-induced bone gains during growth are not maintained throughout old age [264, 414, 602] and that therefore, to be an effective preventive strategy against osteoporosis, exercise should be performed throughout life. For instance, in a study where BMD and fracture rate were assayed in former soccer players and age matched controls, it was shown that despite exercise induced an increase in peak BMD during youth, when activity ceased there was an accelerated bone loss, and at 60 years of age

there were no differences between former athletes and controls regarding BMD and fracture rate.

Despite the extensive evidence showing that exercise benefits bone health [176, 292, 78, 222, 219, 220, 205, 397, 579, 576, 603], observational studies and clinical trials have shown that exercise in the adult has no effects or produces only minor BMD increments [351, 350, 273, 349]. Because osteoporosis is defined in terms of BMD and because DXA derived BMD screening is an easy, minimally invasive and widely available way of estimating bone mass, BMD has been the primary outcome in the majority of the studies for determining exercise-induced improvements in fracture risk. However, regardless of its value diagnosing osteoporosis, BMD increases correlate poorly with fracture risk reduction following treatment [94], which suggests that its usefulness as a surrogate for treatment efficacy may be limited [500]. This is the result of the fact that bone strength depends on several material and structural properties [501] and changes in these properties are not necessarily captured by a shift in BMD [136]. For instance, a meta-analysis about the efficacy of fluoride for the reduction of fractures in osteoporotic post-menopausal women [208] demonstrated that despite the treatment was able to increase BMD by 16% at the end of 4 years, there was no reduction in fracture risk what so ever in the treatment group. Therefore, increased BMD did not resulted in a reduction in fracture occurrence, which in this case might be due to the inability of fluoride to restore trabecular microarchitecture [1] an important determinant of bone resistance. In opposition, post-menopausal woman to witch raloxifene, a selective estrogen receptor modulator (SERM), was given for 3-years, displayed a significant decrease in fracture occurrence despite average BMD increases were only about 2.5%. In another study [488], BMD increases following raloxifene treatment were shown to explain only 4% of the observed vertebral fracture reduction. Consequently, assessing exercise effectiveness in the reduction of fracture risk by monitoring just BMD changes is insufficient and likely misleading and would instead be best achieved by monitoring a broad set of bone properties that might change with treatment reducing thereby fracture risk.

One of the bone properties for which there is growing evidence about its importance for bone health and that is undervalued by bone mass measurements is the viability of the osteocyte network [465]. Osteocytes are the bone resident cells as they are embedded in the bone extracellular matrix. They are derived from osteoblasts that were entombed during the deposition of new bone. Osteocytes contact through cytoplasmatic projections, forming a syncytial network [436] that regulates local bone responses to mechanical (un)loading [618, 217, 544, 543, 305] as well as the systemic mineral homeostasis [66, 252, 513, 50]. Despite being for long disregarded, their role in bone physiology is now evidenced by a wealth of experimental studies showing that osteocytes are essential for the preservation of bone tissue integrity [465] being bone fragility in several cases associated with a decrease in osteocyte viability [343]. Advanced age [571], low estrogen levels [554, 555, 295, 156] and glucocorticoid therapy [399, 437], are all conditions leading to bone fragility which are also associated with loss of osteocyte viability. For instance, studies performed on bone biopsies evidence that osteocyte density is lower in osteoporotic patients than in health individuals [384], and a 34% lower number of osteocytes has been identified in woman who would latter suffer vertebral a fracture [448]. Moreover, studies on experimental animals also demonstrate that targeted osteocyte ablation rapidly leads to a decrease in bone strength [547] showing therefore that the presence of a viable osteocyte network is critical for the maintenance of bone health.

Interestingly, besides detecting and converting mechanical into biochemical signals within bone [285] osteocytes seem to depend on these same mechanical signals for their own survival [438]. For instance, mice subjected to tail suspension show a rapid increase in osteocyte apoptosis [13]. Conversely, studies performed on cultured osteocytes demonstrate that when subjected to mechanical stimulation they have increased viability compared to unstimulated osteocytes [438], and that fluid shear stress increases in osteocytes the Bcl-2/Bax ratio [35], which represents protection against apoptosis. Although the increase in osteocyte viability induced by mechanical stimulation has not been yet confirmed *in vivo*, mechanical stimulation induced by regular physical activity seems to have the potential to counteract osteocyte death

associated with age and estrogen deficiency which is unaccounted by merely performing bone mass measurements thought routine imaging techniques.

Considering therefore the several questions raised, the global purpose of present work is to investigate how ageing and low estrogen levels affect bone tissue quality thereby compromising its mechanical resistance as well as to study how physical inactivity might affect those changes contributing thereby to the increase in bone fragility and fracture risk.

The following work is divided in four main sections. In the first two sections an introduction and a global review of the literature, regarding the physiology and pathology of osteoporosis as well as about the effects of exercise on bone quality, is presented. In the third section the experimental design, the methods used, results obtained and a specific discussion of those results is presented. A global and comprehensive discussion of the results as well as of the main conclusions of the work is presented in the fourth section.

In order to answer the main question that was raised, we performed a series of studies, which are presented in the second section of the work, each one dedicated to answering a specific aspect of the problem. The objective of each of the studies is:

Study 1: To investigate if physical inactivity is a major contributor to menopause related sarcopenia.

Study 2: To investigate if lifelong sedentary behavior aggravates the detrimental effects of age on bone health.

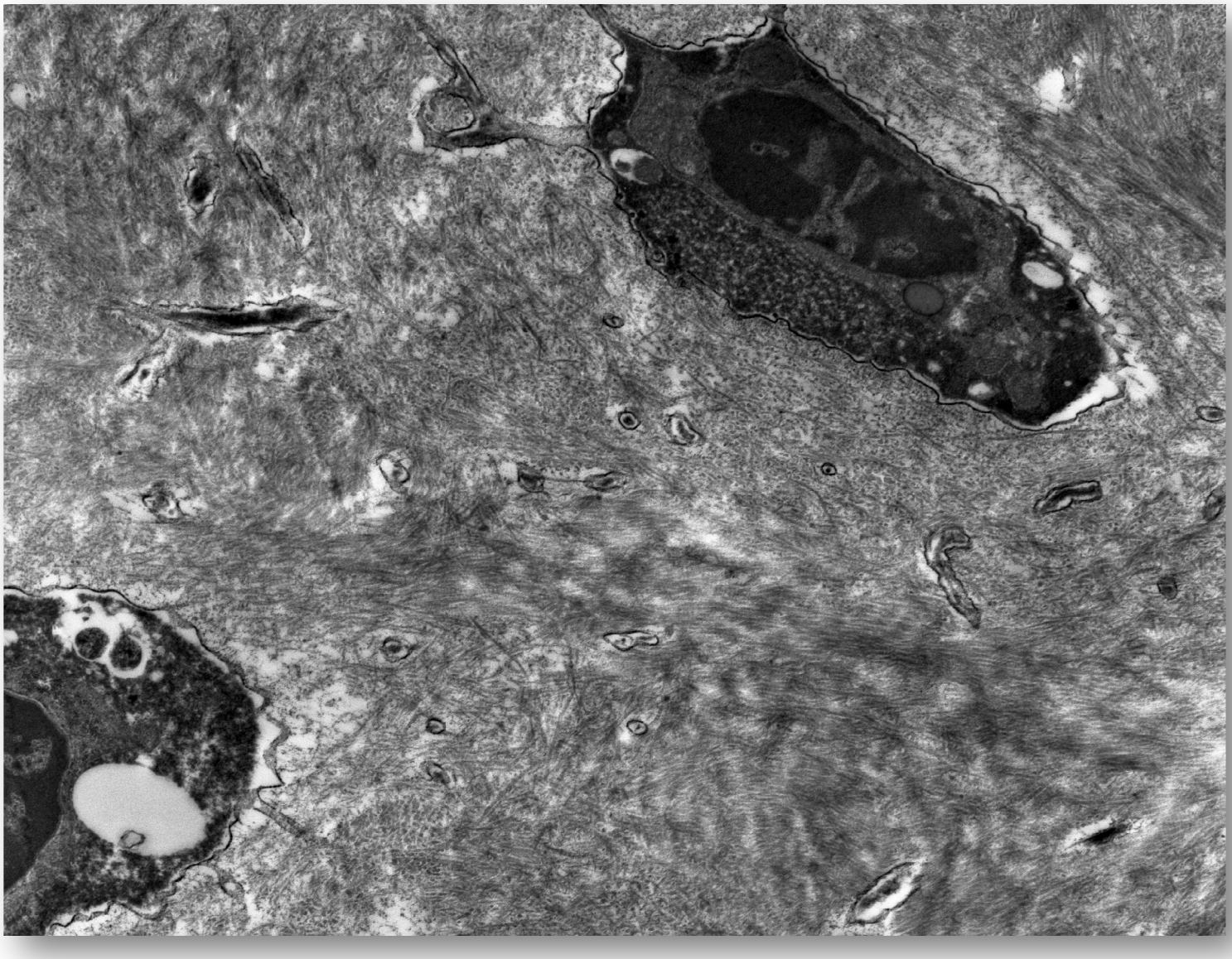
Study 3: To investigate how bone mineral content, trabecular microarchitecture, femoral neck geometry, radiographic absorptiometry and biochemical markers of bone turnover are influenced by estrogen loss and how well are they associated with the proximal femur biomechanical properties.

Study 4: To determine if regular physical activity is able to prevent bone mass losses, mineralization degree losses and to reduce bone resorption rate induced by estrogen deficiency as well as to determine if these changes are captured by radiographic absorptiometry changes.

Study 5: To investigate if *in vivo* mechanical stimulation induced by voluntary exercise reduces osteocyte death following estrogen deficiency with protective effects on bone mechanical properties.

CHAPTER II

State of The Art



1. DETERMINANTS OF BONE STRENGTH AND FRAGILITY

1.1 Bone Physiology

Bones are the individual components of the skeletal system. They accomplish several functions ranging from protection of vital organs, such as the brain or the mediastinum, act as attachment site and lever for muscles and tendons allowing them to produce movement, house the bone marrow and also importantly, they serve as a mineral reservoir, mostly of calcium and phosphate. The vast majority of the body's reserves of calcium are within the bone extracellular matrix (ECM) and when adequate amounts of this mineral are not absorbed by the intestine to meet the organism needs an endocrine response is triggered by the parathyroid glands in order to promote bone resorption, thereby releasing calcium into the circulation and making it available to be used elsewhere in the body [393].

At the microscopic level, bone tissue consists mostly of an ECM made from an organic and a mineral fraction. The organic fraction is composed mainly of type I collagen but also by a variety of other non-collagenous proteins. Deposited in the organic matrix is the mineral fraction formed predominantly by calcium hydroxyapatite crystals [40]. At the macroscopic level, bone tissue can be organized in the form of cortical or trabecular bone. Cortical bone is organized in a compact way forming the outer shell of all bones as well as the shafts of long bones. Trabecular bone is organized in an alveolar fashion forming the interior of irregular bones and the metaphysis and epiphysis of long bones [40].

Bone tissue is continuously remodeled thought life as a result of the action of three specialized types of cells, namely osteoclasts, osteoblasts and osteocytes. Osteoclasts are multinucleated cells derived from the hematopoietic lineage that adhere to the bone matrix forming a sealed compartment between them and the bone surface. They promote bone lyses through the secretion of hydrochloric acid and catabolic enzymes, releasing afterwards the degraded remnants to the extracellular fluid [76]. Osteoblasts are derived from mesenchymal stem cells (MSC), and they are responsible for the synthesis and deposition of the bone ECM, which is first laid as unmineralized bone (called osteoid) and afterwards undergoes progressive mineralization [148]. During

bone formation, some osteoblasts become embedded in the ECM differentiating thereafter as osteocytes, which comprise the vast majority of cells in the bone tissue. Osteocytes are connected between them and with other cells on the bone surface by projections that travel within small channels through the bone matrix called canaliculi, which are filled with extracellular fluid. Damages to the osteocyte cytoplasmatic projections as well as disturbances on their surface caused by the flow of the extracellular fluid in the canaliculi, allow the osteocyte to detect damage to the bone matrix as well as to sense mechanical loading applied to the bone, sending thereafter signals to other bone cells in order to initiate bone remodeling [66]. Bone remodeling (or bone turnover), occurs through life in the various bone surfaces (endocortical, intracortical and trabecular surface) in order to adapt bone mass to its mechanical needs, to repair microdamage and to preserve calcium homeostasis. Remodeling involves the removal of a portion of bone by osteoclasts, followed by the filling of the cavity formed with new bone deposited by osteoblasts [418].

1.2 Pathophysiology of Osteoporosis and Bone Fragility

Osteoporosis is defined conceptually by the World Health Organization (WHO) as low bone mass and microarchitectural deterioration of bone tissue leading to increased occurrence of low trauma fractures [408]. In order to translate this definition to the clinical practice allowing those with osteoporosis to be more accurately diagnosed, an operational definition was established defining that osteoporotic patients are those having a bone mineral density (BMD) that is 2.5 or more standard deviations lower than the average bone mass of healthy young adults [408]. Nevertheless, despite measures of BMD are well correlated with fracture risk at the population level [345, 125], they merely explain a fraction of the individual bone strength. Moreover, there is substantial evidence showing that the majority of fragility fractures occur in individuals who do not have osteoporosis according to the operational WHO definition [496]. For instance, results from a recent study in which femoral strength was assayed by finite-element analysis and dual-energy X-ray absorptiometry (DXA) in individuals ranging from 20 to 90 years old, show that the

decline in femoral neck strength with age is much more pronounced than the decline in BMD, suggesting therefore that the prevalence of low bone strength was significantly higher that the prevalence of osteoporosis [270]. These data show that bone strength evolution with age occurs somewhat independently of the evolution of BMD, and that therefore other bone properties are also of importance for determining fracture occurrence. Several epidemiologic studies also support this finding. A recent study performed with more than 7800 individuals, whose fracture occurrence was tracked for a 7 years period, identified that only 44% and 21% of non vertebral fractures, occurred in women and men with T scores lower than 2.5, respectively [496]. Similar results were also obtained in a different study where fracture occurrence and BMD were followed for 3 years in a population of 16500 women older than 50 years [124]. Again the highest number of fractures occured in women that were not osteoporotic according to the BMD definition. Several other studies also support the notion that low BMD only explains a small portion of fracture risk, and that bone fragility is not just ther result of low BMD [423, 530, 582]. Furthermore, studies with the objective of determining the efficacy of pharmacological treatments in the reduction of fracture risk show that decreases in fracture risk following treatment are disproportionate to changes detected in BMD [126, 103, 589]. BMD changes alone were unable to explain the fracture risk reduction and therapy efficiency, and therefore improvements were the result of changes in other bone properties [435, 434]. Moreover, treatment with fluoride has been shown to increase fracture risk flowing 2-years of treatment, despite significant increases in BMD [373], showing therefore that the use of BMD as a surrogate for treatment efficacy could be extremely deceiving.

1.2.1 Changes in the determinants of Bone Strength in Osteoporosis

Bone strength is the maximal amount of load that a bone can tolerate before it fails due to a fracture [560]. Considering the hierarchical organization of the bone [361] it is currently well-established that several factors, from the collagen properties and matrix mineralization degree to the whole bone geometry, all contribute to bone strength

[347, 501, 224]. Moreover, the development of new biochemical assays and the increasingly higher resolution of imaging techniques, namely microcomputed tomography (μCT) and magnetic resonance microimaging (μMRI), as well as the adaptation of engineering modeling procedures (such as finite element analysis) to bone biology, have allowed to increase the awareness about the importance that several other properties beyond BMD have in determining bone strength. Therefore, impaired bone strength in osteoporosis might result from decreases in the amount of bone mass, decreases in the mechanical properties of the bone tissue, changes in the bone microarchitecture or geometry, or due to a combination of all the previous.

1.2.1.1 Bone material properties

Bones are composed mainly of ECM which is a composite material made up mainly of type I collagen, but also to a minor extent from noncollagenous proteins and proteoglicans [174]. These elements are organized as an organic network, between which small hydroxyapatite crystals are laid down to grow. The two-phase composite nature of bone fabric enables it to absorb loads by elastic deformation but simultaneously to bear high loads before fracturing, being the mineral phase responsible for its stiffness and the collagen fibers for its toughness [501, 174]. Therefore, alterations in either fraction have the potential to affect the bone mechanical properties and thereby the fracture risk.

Type I collagen, laid down by osteoblasts during bone formation, is the major organic constituent of bone tissue. Collagen is first synthesized in the precursor form of procollagen, constituted by three polypeptide chains that are organized in a triple helix structure, and stabilized by post-translational modifications and disulfide bond formation. Following secretion into the ECM the soluble procollagen molecules are cleaved of their N- and C- terminals enabling spontaneous self-assembly into collagen fibrils. These are further stabilized by post-translational modifications that allow the formation of intermolecular and interfibrilar crosslinks in the collagen fibrils through the action of lysyl oxidase which is responsible for the activation of lysine and hydroxylisine residues [578]. The crucial importance of collagen for bone strength is

observed in pathologies like *osteogenesis imperfecta* were mutations affecting collagen genes lead to abnormalities in the collagen polymer and to the formation of a disorganized fibril structure resulting in a substantial decrease in bone strength and increased fracture occurrence [346]. The formation of inter and intrachain cross-links is a key feature for the collagen mechanical properties, since they maintain the several polypeptide chains in a closely organized fibril structure. These cross-links can be enzymatically formed by the action of lysyl hydroxylase or by non enzymatic processes inducing the formation of advanced glycation end products (AGEs) [578]. The most abundant enzymatically derived collagen cross-links are pyridinoline and deoxypyridinoline (collectively called pyridinium cross-links), while pentosidine is the most common AGEs associated cross-link [578]. There is evidence suggesting that changes in cross-linking are associated with reduced bone strength and increased fracture risk [410, 33, 38]. The reduction in pyridinium cross-link content in bone induced by lysyl oxidase inhibition was shown to reduce substantially the bending strength and elastic modulus of bone from experimental animals despite the absence of differences in bone mass [410]. It was also shown, in human bone from different skeletal sites, a relationship between bone strength and enzymatic cross-link profile [33]. In vertebral trabecular bone, the amount of pyridinoline cross-links was also shown to correlate with the bone ultimate strain while the ratio pyridinoline/deoxypyridinoline was shown to correlate with the bone ultimate stress and elastic modulus [38]. Enzymatic cross-link formation and collagen assembly [378] is also regulated by non-collagenous proteins present in bone matrix. These have also been shown to significantly influence the collagen mechanical properties [585].

Glycation is a common posttranslational modification of proteins mediated by reactive aldose or ketose sugars and other metabolic intermediates that can react with free amino groups in lysine, hydroxylysine or arginine residues, forming in some cases adducts to proteins while in others promote protein cross linking [265]. Non enzymatic cross-link formation due to glycation is shown to be associated with the deterioration of bone mechanical properties. Several *in vitro* studies demonstrate that an increase in AGEs in bone increases the tissue brittleness, leading to microdamage accumulation

and decreases in bone strength [183, 484, 570]. The formation of AGEs associated cross-links is also thought to be the main responsible for the increased fracture risk and bone fragility seen in diabetic patients, especially in type II diabetes which even often have increased BMD [575, 483]. This hypothesis however remains controversial since the altered biomechanical properties of bones from diabetic patients may be related to a variety of factors, namely hormonal and vascular factors, bone structure and density, and other matrix components [497]. There is also evidence showing that bone matrix composition in the osteoporotic bone is different from bone of healthy individuals, which could also affect its mechanical properties. For instance, a study by Bailey and colleagues [34] has reported that bones from osteoporotic patients had an altered expression of type III and IV collagen when compared to bones from individuals without osteoporosis. However, the major differences reported between the osteoporotic and normal bone are in terms of collagen hydroxylation and cross-link formation. For instance, in a study where type collagen I was isolated from the femoral heads of osteoporotic women, it was shown a significantly higher degree of hydroxylation of lysine residues in osteoporotic bone compared to normal bone [297]. Recently, a study using infrared imaging analysis of iliac crest biopsy specimens from patients with osteoporotic and multiple spontaneous fractures also showed that collagen cross-links in newly deposited bone was markedly different between patients with osteoporosis and normal controls [422].

The inorganic fraction of bone tissue is also of crucial importance for its mechanical properties, being the degree of mineralization the main responsible for the bone stiffness [501, 174]. However, not only the degree of mineralization matters as also the individual characteristics of the hydroxyapatite crystals, namely their size and shape. Indeed, results suggest that adequate bone tissue mechanical strength is achieved with the presence of mineral crystals with different sizes as bones from older and osteoporotic animals are shown to have only larger size mineral crystals while younger animals, with increased bone strength, are shown to have a greater variety of crystal sizes [71]. In addition, in a study with human bone samples from donors ranging between 52 to 85 years old, it was also possible to identify an overall reduction in

crystal heterogeneity with age [613]. As mineral crystals grow between the collagen fibrils [173] it is possible that crystal overgrown may induce physical damage to the adjacent collagen fibers thereby affecting the tissue mechanical properties. Interestingly, studies performed in monkeys showed that the administration of parathyroid hormone (PTH) was able to enhance bone mechanical strength which was associated with an increase in the variety of bone tissue mineral crystal size [421]. As PTH increases bone turnover [278], it is most likely that this increase has promoted the removal of older bone with larger mineral crystals, which was then replaced by new bone tissue with smaller mineral crystals, increasing thereby crystal size heterogeneity.

The degree of crystallinity (the orderliness of the crystal minerals) assayed in human cadaveric femurs, has also been shown to explain between 6.7% and 63.5% of the variation in the bone mechanical properties, with crystallinity increases being associated with increases in bone strength and stiffness [612]. Despite the apparent randomness of mineral crystal growth in bone, there is evidence showing that this is a tightly regulated process where many factors may contribute to the formation of the mineral crystals or in opposition may inhibit their formation [181, 9, 574]. Therefore, by influencing crystal growth and maturation, the presence of some proteins appears to be critical in determining the inorganic properties of the bone tissue and consequently its mechanical properties [29, 11, 10]. Hence, changes with age in the secretion of proteins that might be involved in biomineralization [142] could lead to modifications of the mineral crystal growth pattern.

1.2.1.2 Cellular density

Osteocytes were for long considered as just terminally differentiated osteoblasts that remained in a somewhat quiescent state after becoming entombed in the mineralized matrix during the deposition of new bone. Most certainly due to both their apparent inactive state and to the difficulty in obtaining osteocytes in reasonable numbers and purity from experimental animals, their function remained largely unknown, and therefore undervalued, for long. The establishment of cell lines [65, 267] able to mimic the features of primary osteocytes enabled them to be studied *in*

vitro. There is now a wealth of experimental evidence showing that osteocytes are absolutely essential for the preservation of bone tissue integrity and that bone fragility is in several cases triggered by a decrease in osteocyte viability.

Several studies have verified that triggering osteocyte apoptosis, whether by mechanical [301] or biochemical [151] insults leads to the activation of osteoclasts into that site, increasing local bone resorption. Although the precise mechanism leading to osteoclast activation remains largely unknown, there is evidence suggesting that decreases in transforming growth factor beta (TGF-β) [217], increases in receptor activator of nuclear factor kappa-B ligand (RANK-L) and macrophage colony stimulating factor (M-CSF) [301] as well as the formation of apoptotic bodies [290] are all involved in osteoclast activation following osteocyte apoptosis. For instance, it was previously shown that cultured osteocytes were able to stimulate osteoclast formation from bone marrow stem cells, through the expression and secretion of M-CSF and RANK-L [628], which are growth factors required for osteoclast commitment and activation [76]. Moreover, mechanically damaged cultured osteocytes were also shown to stimulate the activation of osteoclasts in a closely restricted area by releasing M-CSF and RANK-L [301]. A recent study with experimental animals also demonstrates that microdamage to the bone matrix was able to trigger osteocyte apoptosis which was spatially and temporally linked to osteoclast activation and intracortical bone remodeling [90]. Most interesting however was that blocking osteocyte apoptosis was able to prevented the osteoclast activation [90]. Another recent study [290], also provides evidence that the increased osteoclast activation and bone resorption that follows osteocyte death is signaled through the release of apoptotic bodies independently of RANK-L and M-CSF signaling [290]. Interestingly apoptotic bodies derived from osteoblasts were unable to promote osteoclast activation either *in vivo* or *in vitro*, which highlights the specific importance of osteocytes in the mediation of localized osteoclast activation [290].

Osteoclast activation seems therefore to be achieved by the loss of the constitutive inhibition of osteocytes over osteoclasts [203] as well as by the release of stimulatory factors following their death [301, 290]. If restricted to particular bone regions this must be seen as a protective mechanism, as it promotes the renewal of damaged bone

with worse mechanical properties [90]. However large increases in osteocyte death could lead to excessive bone resorption and increased intracortical porosity, ultimately compromising bone strength [547]. Osteocytes are therefore essential for promoting targeted remodeling of bone tissue and impairment of the osteocyte network may affect bone quality by hindering the repair of damaged bone [149] or by promoting excessive bone resorption. *In vivo* studies performed on iliac bone biopsies also show that there is a strong association between osteocyte density and fracture occurrence. Qiu al colleagues [448] identified a 34% lower osteocyte density in women who latter would suffer spontaneous vertebral compression fractures demonstrating hence that this was an important factor influencing bone quality. Moreover, targeted ablation of osteocytes in experimental animal was also shown to lead to decreases in bone strength [547]. Aging [571], lack of estrogen [156] and glucocorticoid therapy [399, 437], are all causes associated with loss of osteocyte viability that also lead to bone fragility. Moreover, lack of mechanical stimulation is also shown to trigger osteocyte apoptosis resulting in the rapid increase in bone resorption seen for instance in tail suspended mice [13].

Although many of the effects of osteocyte apoptosis on bone fragility are recognized to derive from their direct action over osteoblasts and osteoclasts, a connection between the decrease in osteocyte density and loss of bone strength due to hydration status is also acknowledged. It is broadly described that water is responsible for some hydraulic properties in hard tissues, and that dehydrated structures have increased brittleness [377]. Bone is a hydrated structure [601] and much of its water is estimated to reside in the canalicular system due to the presence of a glycoprotein and proteoglican rich pericellular matrix surrounding the osteocyte body and its dendritic projections [619]. The death of osteocytes and the disruption of their associated pericellular matrix could therefore affect the bone hydration status compromising bone strength [594]. In fact, there is some evidence that bone hydration decreases with age [234] which is also consistent with an osteocyte density decrease. Finite element analysis modeling shows evidence that hydration is able to increase 2.5 fold the cortical bone ultimate strength [322] and direct mechanical testing of cortical

bone specimens with different degrees of hydration show that dehydration progressively increases bone tissue brittleness and decreases its strength [398].

1.2.1.3 Bone turnover

The existence of an adequate balance between bone formation and resorption is crucial for bone quality since the uneven increase in resorption leads to bone loss. However, it is important that bone turnover also occurs at an adequate rate. Several clinical trials have demonstrated the importance of bone turnover in skeletal fragility. For instance, in a recent study with post-menopausal women, fracture occurrence was shown to be associated with the rate of bone resorption independently of other fracture risk predictors, such as BMD and previous factures [521]. In another study in women undergoing treatment with raloxifene, changes in bone turnover markers, but not in BMD, were associated with a reduction in vertebral fractures.

Bone strength depends, in a great extent, on the degree of tissue mineralization, which in turn is greatly determined by the rate of turnover [64]. During the formation of new bone, osteoblasts secrete the organic matrix that initially serves as a scaffold for the initial mineral templates to be formed (primary mineralization). Following this initial period, the new bone progressively suffers further mineralization (secondary mineralization), with a slow and gradual increase in mineral crystal number and size [194]. If bone turnover rate if too high, secondary mineralization does not occur efficiently, as this slow process does not have the enough time to occur before a new remodeling unit reabsorbs the new bone again, leading to a decrease in the overall bone tissue degree of the mineralization and consequentially to a decrease in bone stiffness [63, 62, 61].

Conversely, excessively reduced bone turnover rate leads to a decrease in the renewal of bone tissue and therefore to the accumulation of older and more extensively mineralized bone tissue [62, 372], which has two main biomechanical disadvantages. Firstly, more extensively mineralized bone becomes more brittle [129], and therefore is unable to absorb energy by elastic deformation. Consequently, loads applied during everyday movements will be dissipated through structural failure,

initially by the development of microcracks that eventually will progress until reaching complete fracture. Secondly, one of the main purposes of bone turnover is to selectively remove damaged old bone and to replace it by new, more mechanically competent bone. An excessively low bone turnover rate leads therefore to the accumulation of damaged bone [352, 353], with reduced elastic properties [86] increasing the probability of microcrack proliferation and fracture occurrence. When turnover is too much suppressed, not only new microcracks accumulate, as they have more opportunity to grow to critical sizes. Over suppression of bone turnover during long-term use of anti-resorptive therapies has been associated with spontaneous fracture occurrence and absence of fracture healing [401]. An adequate rate of bone turnover is therefore necessary for achieving a properly mineralized bone structure that best combines stiffness and brittleness, since undermineralized bone tends to be very weak and overmineralized bone too brittle.

1.2.1.4 Microarchitecture

Due to the greater surface per volume ratio, trabecular bone is rapidly affected by increases in bone resorption. Individual trabeculae become progressively thinner while trabecular separation increases, shifting from a plate-like shape to a rod-like shape. Progressive perforation of individual rods leads to loss of trabecular connectivity and reduces the number of trabeculae, resulting ultimately in trabecular architecture deterioration [514, 303, 74]. Since at several skeletal sites the majority of bone is trabecular, like in the extremities of long bones and vertebral bodies, these progressive changes in trabecular architecture rapidly lead to compromised strength in these regions [558, 232]. In a study where the contribution of trabecular bone mass and architecture to the compressive strength of rat vertebra were assayed, it was shown that they were able to explain 91% of the compressive strength variability [67]. In another recent study [590], trabecular bone volume and trabecular microarchitecture of the distal radius and tibia of postmenopausal women were shown to explain 96% of the mechanical properties variation. However, in another study, trabecular bone volume was only able to explain between 37 to 67% of the bone

mechanical properties [99]. Trabecular bone architecture has also been demonstrated to be a determinant of bone strength independently of BMD [315]. Moreover, regional variations in trabecular architecture between different sites of the same vertebra have also shown to be correlated with the site of fracture and with the failure load after compression testing [522], while BMD was shown to be weakly correlated [523].

As cortical bone represents a substantial amount of the total bone mass, and it is responsible for substantial load bearing specially in the appendicular skeleton bones [334], cortical bone architecture is also a significant determinant of bone quality and therefore of bone strength [49]. The imbalance in bone resorption seen in osteoporosis also leads to cortical bone architectural changes, most frequently to the progressive increase in intracortical porosity. This progressive increase leads to cortical bone trabecularization witch consequentially compromises bone strength [511, 119]. For instance, biomechanical studies performed in cortical bone samples of human femur show that bone stiffness is inversely correlated with the porosity of cortical bone [146]. Moreover, in a recent study it was shown that intracortical porosity was responsible for a 6% decrease in tibia stiffness and to a significant load transfer from the cortical compartment to the already weakened trabecular bone compartment [85]. Together these results demonstrate that microarchitectural alterations that occur either in cortical or in trabecular bone are responsible for significant changes in the bone mechanical properties.

1.2.1.5 Bone geometry

Several parameters of bone geometry, namely size, shape, cortical thickness and cross sectional area have revealed a significant relationship with bone strength and fracture occurrence. For instance, in a recent study were 677 young men were examined at the age of peak bone mass for bone geometry, BMD and fracture occurrence, it was shown that fractures were more frequent in those with decreased cortical thickness and larger endosteal perimeter but were not significantly associated with cortical BMD [540]. Moreover, several studies performed with the objective of testing the effects of pharmacological treatments for osteoporosis have shown that

changes in cortical bone geometry, like increased cortical thickness and increased cortical perimeter are associated with increased bone strength and reduced fracture occurrence [24, 152, 26]. Differences in bone strength between african and caucasian post-menopausal women [391, 390], as well as between aged men and women [478, 479], have also been attributed to differences identified in the cortical bone geometry of the femoral shaft, namely to its cross sectional area. The increase in cortical perimeter is a crucial geometric parameter influencing bone strength since, as mechanical engineering shows, increasing a hollow cylinder diameter provides exponential resistance increases to bending and torsion without necessary increases in bone mass [560]. In fact, some authors have argued that the increase in bone diameter seen in cases of age or menopause associated bone loss are the result of a compensatory mechanism for the decreases in bone mass and trabecular architecture, helping in this way to maintain bone strength [479, 499, 409]. Therefore, it could be possible that the major adaptations in bone geometry seen in bones that have the greater deterioration in other parameters of bone quality are in fact signs of weakened bones.

There is also growing awareness about the importance of femoral neck geometry for bone quality, despite the existence of conflicting results regarding the different parameters ability to predict fracture risk independently of BMD. This knowledge is leading to the development of DXA scanners software that provides additional information about bone geometry, namely about the hip-axis length, femoral neck shaft angle, femoral neck cross-sectional area and moment of inertia, and to the computation of equations that based on these parameters better predict the risk of fracture [164, 515, 46]. The combination of both BMD and upper femur geometrical parameters has already been demonstrated to improve the assessment of hip fracture risk provided by BMD alone [446], demonstrating the importance of proximal bone geometry for femoral strength and for the risk of fracture occurrence. In a study with the objective of determining the relationship between femoral neck geometry and the risk of hip fracture in post-menopausal women [153], it was identified that hip fractures were more frequent in individuals who had greater femoral neck width,

femoral shaft width and femoral neck axis length. In other studies, hip axis length has also been found to be significantly longer in women that had suffered a hip fracture compared to women without previous fractures [175, 53]. African women also have smaller bone widths and hip axis lengths compared to caucasians and these geometrical differences are thought to be also associated with hip fracture occurrence differences between these two populations independently of BMD differences [551]. Despite these evidences, others have not identified a relationship between femoral neck axis length and fracture risk [374]. Neck-shaft angle and femoral neck width are geometric parameters that are also found to be increased in individuals with greater fracture risk [22]. Regression analysis has showed that an increase of 1 standard deviation in neck-shaft angle was associated with an increase in 2.45% and 3.48% increase in fracture risk in men and women respectively, while the increase in femoral neck width was associated with an increase in 2.15% and 2.40% fracture risk in men and women, respectively [22]. Neck-shaft angle and femoral neck width were however not found to be significantly associated with hip fracture risk in another study, while an increase in each standard deviation in hip axis length doubled the risk of hip fracture independently of age and of bone mineral density [163]. The magnitude and direction of forces acting on the femoral neck after a sideways fall is also determined by the geometry of the proximal femur. Considering that a significant number of hip fractures occur as a sequence of a fall [420], it was found that individuals that have a longer femoral neck moment arm have more frequent hip fractures after a sideways fall due to a greater concentration of forces in the femoral neck [588]. Most interestingly, a recent study has identified an association between genetic polymorphisms involved in the bone mineralization pathway and several femoral neck geometrical parameters, suggesting that both traits are interconnected by genetic influence [108]. Anti-resorptive and anabolic agents used for the treatment of osteoporosis have also been shown to modify proximal femur geometric parameters increasing thereby bone strength [199, 567].

1.2.2 Mechanisms leading to increased bone loss and skeletal fragility in Osteoporosis

Although an extensive number of heterogeneous causes might lead to bone loss and increased skeletal fragility [210], age and most importantly estrogen deficiency, are the main causes of osteoporosis [456, 486]. Currently, the most broadly accepted explicative model for osteoporosis considers estrogen deficiency as the main cause for the rapid bone loss seen in woman following menopause as well as the primary cause for the slow bone loss observed with increasing age in both women and men [456, 279]. The relevance of estrogen in the maintenance of bone mass in men is documented in several studies. For instance, absence of estrogen receptors was shown to result in reduced bone mass in a man that had normal testosterone levels [519]. Moreover, men with mutations on the enzyme aromatase, which is responsible for the conversion of testosterone into estrogen, also have reduced bone mass. Nevertheless, bone mass in these cases was restored by administration of estrogen [89]. In addition, several studies have shown that with age there is a significant decrease in the available estrogen levels in men [279, 517, 198], and that both in young and in aged men, estrogen levels rather than testosterone levels are a major predictor of bone mass [279, 517, 198].

More recently, improvements in the knowledge about the cellular mechanisms associated with bone loss have called attention to the importance of ageing dependent bone cells damage in the pathogenesis of osteoporosis [340]. Although this awareness has shifted the cornerstone of the osteoporosis cause away from estrogen, it is seen as a complementary rather than an opposite view about the pathogenesis of osteoporosis as estrogen deficiency and changes in bone cells and ECM due to age are most likely to be cumulative [280].

The rapid bone loss observed in postmenopausal women is associated with the abrupt decline in ovarian hormone production. Despite the evidence that decreases in trabecular bone volume begin, at some skeletal sites, before the menopause [458], most studies show that until this period BMD remains relatively constant and that fracture rates are low [457]. However, after the menopause onset, fracture rates

increase exponentially as a consequence of bone mass loss, disrupted trabecular microarchitecture and decreased bone tissue material properties [486]. The onset of estrogen deficiency leads to a significant increase in bone turnover with resorption greatly exceeding formation [627, 185]. This increased and unbalanced bone remodeling results in an initial phase of rapid bone loss [74] and deterioration of trabecular architecture [88], followed by a phase of slower bone loss [318], similar to what is observed in men [457]. The loss of bone mass following estrogen deficiency results both from the direct effects of low estrogen on bone cells activity as well as from its effects on other organs that regulate calcium homeostasis leading thereby indirectly to increased bone resorption. Nevertheless, it is possible that other factors, such as a reduction in mechanical stimulation due to a decline in physical activity levels may also contribute to this decrease in bone mass and the increased skeletal fragility.

1.2.2.1 Direct role of estrogen on bone cells

Estrogen is a global classification for several steroid and non steroid compounds that are able to induce estrous cycle. The three major naturally occurring estrogens are estrone, estriol and predominantly estradiol (17β-estradiol). Estrogen is able to exert direct effects on bone cells due to the presence of estrogen receptors (ER) on osteoblasts [137, 158], osteocytes [621] osteoclasts [407] and in bone marrow MSC [403].

One of the effects of estrogen is the regulation of osteoblasts differentiation [132]. Both osteoblasts and adipocytes share the same MSC progenitors [376]. Several studies show that estrogen is able to promote osteoblast differentiation in detriment of adipocyte differentiation from their common precursor increasing this way the number of differentiated osteoblasts in bone. Dang and colleagues [132] showed that 17β-estradiol stimulated the differentiation of both cultured MSC and mice bone marrow cells into osteoblasts and inhibited adipocyte formation. Similar results were also reported in another study [403] where bone marrow cells treated with estrogen showed increased alkaline phosphatase (ALP; a specific osteoblast enzyme) activity and decreased lipid accumulation, which were reversed by administration of an ER

antagonist. These results demonstrate that estrogen directly influences osteoblast differentiation and that estrogen absence leads to a shift towards adipocyte differentiation, which explains the accumulation of adipocytes in the bone marrow of osteoporotic patients bone [253, 614], and its decrease following estrogen therapy [537, 155].

Estrogen is also shown to increase osteoblast proliferation [493], as evidenced by the increased number of cultured osteoblasts from rat bone marrow treated with 17β-estradiol [180]. However, there are also conflicting results showing an inhibitory effect of estrogen on osteoblast proliferation [460]. For instance, estrogen loss is shown to increase osteoblastogenesis [245] while estrogen administration to OVX animals [139] and postmenopausal women [573] is shown to decrease osteoblast number and activity. The reason for the different findings between the *in vivo* and *in vitro* studies seems to reside on the stimulatory effect that estrogen loss has on osteoclast development, activity and survival [508, 257, 526].

A key step in the differentiation of the osteoclast is the activation of RANK by RANK-L [302], which is expressed by bone marrow MSC, osteoblasts and lymphocytes [595, 259, 308, 580]. The interaction between RANK and RANK-L is regulated by a decoy protein secreted by osteoblasts, called osteoprotegerin (OPG), that binds RANK-L preventing its interaction with RANK [512, 566]. There is evidence suggesting that estrogen reduces osteoclast differentiation by increasing OPG secretion by osteoblasts [69, 226, 481] which leads to a dramatic increase in the number of osteoclasts following estrogen loss. Moreover there is also evidence that estrogen induces osteoclast apoptosis [257, 229, 388]. So, estrogen deficiency enhances both osteoclast activity and survival increasing the amount of bone resorbed by each osteoclast, leading thereby to a dramatic elevation in bone resorption. Since the activity of osteoclasts and osteoblasts is tightly coupled [348], an increase in osteoclast activity eventually stimulates an increase in osteoblast activity [245]. The increase in the activity of osteoclasts and the higher bone resorption following estrogen loss therefore leads to an increase in the proliferation of osteoblasts, which explains the contradictory findings between *in vivo* and *in vitro* studies about the effect of estrogen

on osteoblasts. Increases in bone formation following estrogen loss are however insufficient to compensate the increases in bone resorption [295], leading to the unbalance between the two process and consequently to bone loss [606]. In addition, estrogen is also shown to prevent the death by apoptosis of MSC [629], osteoblasts [120, 193] and osteocytes [202, 554, 555].

1.2.2.2 Role of proinflammatory cytokines in bone loss

Increased bone resorption is a frequent complication in periodontal disease [548] and orthopedic implant loosening [17], resulting from inflammation and an increase in the local proinflammatory cytokine production. There is evidence suggesting that this mechanism might be also involved in the pathogenesis of osteoporosis as low estrogen levels are associated with increases in proinflammatory cytokine production which is reversed by estrogen administration [412, 3]. For instance, OVX women showed as soon as 1 week after surgery increases in granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) which were accompanied by increases in biochemical markers of bone resorption in the urine [412]. Interestingly, estrogen administration was able to reduce the levels of proinflammatory cytokines and markers of bone resorption.

There is evidence that the activity of osteoclasts is regulated by several cytokines, namely IL-1, IL-6, IL-7 and TNF-α, and that the secretion of these cytokines is in turn influenced to a great extent by estrogen levels [342, 341]. For instance, OVX leads to a rapid increase in IL-1 and TNF-α levels in the bone marrow of experimental animals which is followed by a decrease in bone mass. However, treatment with IL-1 and TNF-α antagonists completely prevents those bone losses [283]. Additionally, mice lacking IL-1 receptor do not exhibit OVX-induced bone loss [326], suggesting therefore that IL-1 has an important role in mediating OVX-induced increases in bone resorption. TNF-α levels are also increased in the bone marrow of OVX animals and in cultured blood cells from postmenopausal women [412, 450] and there is evidence that estrogen inhibits TNF-α production by T-lymphocytes [527]. Moreover, TNF-α is shown to induce RANK-L expression by MSC [591] and T-lymphocytes [227, 467]. Interestingly, OVX-

induced bone losses are prevented in mice lacking T-lymphocytes [96] and TNF-α [467, 23]. The main effect of low estrogen levels however, appears to be the increase in the number of activated T-lymphocytes in the bone marrow that actively secrete TNF-α [467] being this a consequence of an increase in IL-7 [480, 596] and in interferon-gamma (INF-γ) levels [95].

Several studies have also provided evidence about the involvement of IL-6 on the increased bone losses following estrogen deficiency. IL-6 has been shown to be able to stimulate osteoclast differentiation [327] by acting on osteoblasts or MSC [565]. Moreover, IL-6 expression increases with estrogen loss and decreases with estrogen replacement therapy [191]. Moreover, increases in osteoclast numbers in mice following OVX are prevented by IL-6 inhibition [244]. Additionally, some [441] but not all [282] studies have shown that IL-6 deficient mice are resistant to OVX-induced bone loss. In addition to stimulating osteoclast differentiation, proinflammatory cytokines are also shown to inhibit osteoblast differentiation, further contributing to the uncoupling between bone resorption and formation observed following estrogen loss [596, 189].

1.2.2.3 Oxidative Stress

Reactive oxygen species (ROS) include a variety of molecules, such as superoxide anion, hydroxyl radical and hydrogen peroxide, which can be either generated exogenously or produced within the cell from various sources, being the most relevant the mitochondria electron transport chain [168]. ROS are to a great extent neutralized by antioxidant defenses, which include a series of enzymatic scavengers and other molecules with anti-oxidant properties [168]. The unbalance between the production of ROS and the antioxidant defenses leads to an increase in oxidative stress which may result in damages to a variety of cellular structures such as DNA, proteins and lipids. If the cell is unable to repair or replace the damaged structures, its normal function or even its survival will be compromised.

Increased oxidative stress is thought to be the main driving force of ageing as it has been shown to contribute to age related changes in many tissues [37, 168, 190, 328],

including bone [21], namely by decreasing bone formation [379, 32] and by increasing bone resorption [186, 31, 532]. For instance, cultured osteoblasts and bone marrow MSC exposed to hydrogen peroxide showed an increased oxidative damage and a decreased expression of markers of bone formation such as ALP [379, 32]. Likewise, exposure to hydrogen peroxide is also shown to increase apoptosis in cultured osteocytes [338]. Conversely, increasing ROS in rat calvarial organ cultures [186] as well as in human marrow cultured cells [31] causes an increase in the number of active osteoclasts and enhances bone resorption. In addition to these *in vitro* evidences suggesting that ROS have an important role modulating bone cells survival and activity, clinical studies have also shown evidence of ROS involvement in bone loss. For instance, antioxidant defenses are shown to be decreased in individuals with osteoporotis [336] and the levels of oxidative stress biomarkers are shown to be inversely associated with BMD measurements in both women and men [44, 609].

Interestingly, the increase in ROS with age seems to result, at least in part, of a decrease in estrogen levels, as estrogen is shown to have anti-oxidant effects on bone tissue [21]. For instance, animal studies show that OVX results in the increase in ROS production [387], and that this increase is reversed by the administration of either estrogen or anti-oxidants [21]. Additionally it has also been shown that administration of anti-oxidants prevents OVX-induced bone loss and that the blockage of endogenous anti-oxidants leads to bone loss [307]. Studies on post-menopausal women also showed that oxidative stress is directly related with bone resorption and inversely related with BMD [31].

1.2.2.4 Secondary hyperparathyroidism

The skeleton has a pivotal role in the maintenance of circulating calcium levels, acting as a reservoir from witch calcium is drained in response to states of hypocalcemia. Therefore, reductions in circulating calcium levels, either due to a decreased intestinal absorption or due to an increased renal excretion, will inevitably have consequences on bone metabolism [40]. There is evidence suggesting that low

estrogen levels might be associated with changes in the calcium balance due to increases in calcium lost in the urine as well as in the faeces [279].

Rat intestinal epithelial cells express ER [553] and they are shown to increase calcium transport when stimulated with 17β-estradiol [27] suggesting therefore that estrogen directly stimulates intestinal calcium absorption. Moreover, significantly lower intestinal calcium absorption was observed in OVX rats compared to pair-fed sham-operated (SHAM) counterparts, despite absences in circulating vitamin D differences [400]. In addition, when OVX animals were treated with estrogen, there was a significant increase in intestinal calcium absorption [400, 115]. The action of estrogen in intestinal calcium absorption appears to be mediated by its binding with ER as estrogen induced increases in intestinal calcium absorption are inhibited by the administration of ER antagonists [550]. Moreover, there is evidence that activation of ER increases the expression of calcium transport protein 1, increasing thereby the calcium uptake by the intestinal epithelial cells [568].

The kidney also has a major role in the regulation of circulating calcium concentration by increasing or decreasing tubular reabsorption in a process that involves calcium uptake at the distal tubule luminal membrane, intracellular calbindin-dependent storage and trafficking and finally extrusion through the basolateral membrane by the plasma membrane calcium pump (PMCA) in a way that is closely regulated by Vitamin D [70, 248]. Like in the intestine, there is evidence that the kidney also expresses ER [207] and that they regulate the expression of several genes [241], and it is possible that some of those genes might be involved in the renal calcium reabsorption. For instance, cultured renal tubule cells supplemented with 17β-estradiol have an increase in PMCA activity that is blocked by the addition of an estrogen antagonist [141]. Studies on elder women also show the existence of an inverse relationship between plasma estrogen concentration and renal calcium excretion [140]. Therefore, as estrogen deficiency seems to increase calcium wasting through the kidney, a compensatory increase in bone resorption occurs through an increase in PTH in order to maintain calcium concentration. For instance, serum PTH levels and bone resorption markers are shown to be closely associated and to increase

with age [278]. Moreover, intravenous calcium administration is able to suppress PTH increases and to reduce bone resorption in elder women [312], suggesting therefore that the increased bone resorption in the elder is mostly due to hyperparathyroidism. In men, similarly to what occurs in elder women, there is also evidence of an increase in PTH levels and bone resorption markers with age [157], suggesting that hyperparathyroidism, due to the effects that low estrogen levels produce on extraskeletal tissues, is a likely cause for the progressive bone loss observed in elder women and men [279].

However, bone losses due to hyperparathyroidism appear to assume an important role only in the elder women and men, and not during the early phase of the menopause which is characterized by a rapid bone loss. During this phase, the increased renal calcium excretion and decreased intestinal calcium absorption are a consequence and not the cause of the increased bone resorption. In early post-menopausal women, the high bone resorption induced by the direct effects of low estrogen levels on bone cells, leads to an increase in circulating calcium levels, which consequently results in a compensatory increase in the renal excretion of calcium and a reduction in its intestinal absorption, in order to prevent hypercalcemia [6, 395]. So, as calcium levels remain within normal limits, PTH concentration does not increase in early postmenopausal women [444].

1.2.2.5 Reduced mechanical stimulation associated with physical inactivity

The effects of mechanical stimulation or its lack on bone mass are well described [411]. Exposure to low gravity environments, prolonged bed rest or sedentary behavior, are causes leading to bone loss and compromised bone quality [541]. As there is evidence from studies on experimental animals as well as on humans, suggesting that estrogen might influence motor activity, it is possible that lack of mechanical stimulation caused by decreased physical activity also contributes to the development of bone loss. Physical activity levels in the female rat are shown to vary in accordance to the estrous cycle, with higher physical activity levels being observed during the phases of higher estrogen levels [605]. In addition, female mice that

undergo OVX experience a significant decrease in the amount of voluntary activity, which is reversed by estrogen replacement [196]. Similarly, there is also evidence that the menopause transition in women is accompanied by a decrease in physical activity and energy expenditure [440]. Moreover, post-menopausal women undergoing estrogen therapy have a higher maximal oxygen uptake and anaerobic threshold than post-menopausal women not receiving estrogen [453] suggesting that they are more physically active. These effects are apparently gender independent since aromatase-deficient male mice also show reduced spontaneous physical activity levels [250] while administration of estrogen to castrated male rats significantly increases their physical activity levels [371].

Decreases in physical activity following estrogen loss might be due to the effects that low estrogen concentration has on skeletal muscle, leading thereby to the inability to perform physical activity. For instance, there is evidence that OVX mice have a significant decrease in skeletal muscle contractility [381], that is reversed by estrogen replacement [380]. Moreover, a decrease in *plantaris* muscle type IIx myosin heavy chain (MHC) isoform expression has also been described in OVX rats, but not in OVX rats undergoing estrogen supplementation [432]. OVX has also been shown to induce a shift from type II to type I MHC isoforms in the rat *soleus* and *extensor digitorum longus* muscles [254].

Estrogen also seems to be able to influence voluntary physical activity levels by acting directly on central nervous system structures. For instance, targeted inhibition of ER in the ventromedial nucleus of the rat hypothalamus leads to significant decreases in daily physical activity levels, which are comparable to decreases observed following OVX [386]. Moreover, direct estrogen administration to the medial preoptic area of OVX rats is able to restore physical activity levels [161]. Damages to the anterior hypothalamic nucleus and medial preoptic area also impair the increases in physical activity observed in OVX rats treated with estrogen. There is therefore a significant amount of evidence suggesting that estrogen loss is accompanied by a considerable decrease in physical activity levels, which could thereby contribute to decreases in both skeletal muscle mass and bone tissue mass. Considering the

importance of mechanical stimulation in the preservation of bone mass, it is possible that the progressive loss of estrogen with age might be associated with decreases in physical activity levels which further enhances bone mass losses observed with age.

2. PHYSICAL ACTIVITY AND BONE STRENGTH

The notion that bone structure has the ability to adapt itself to the mechanical forces that are applied to it was established for more than a century ago by the work of Julius Wolff, in what was to became known as the *Wolff´s Law* or *Law of Bone Remodeling* (for reference see [428]). As an orthopedist surgeon, Wolff was able to conduct a series of observational studies in which he described that the variations of bone geometry and trabecular architecture were in accordance with the loads that were applied to the bone [230]. Since that time, several observational and experimental studies have been performed showing that bones are able to increase their mass when subjected to mechanical loading and also that they undergo significant bone mass losses when subjected to unloading, supporting therefore the initial observations made by Wolff that bone tissue is sensitive to mechanical stimulation [222, 219, 220, 205, 397, 579, 576, 603].

2.1 Evidences on athletes

Several observational studies have shown that bone mass is higher in athletes when compared to sedentary individuals [109, 534, 219, 220, 603]. For instance, male professional soccer players were shown to have a significantly higher BMD than age matched sedentary controls. Furthermore, bone mass increases in the lower limbs were shown to result from both a significant increase in bone size as well as in BMD [603]. Comparison between weightlifters and sedentary controls has also been able to demonstrate that bone mass at the lumbar spine and femoral neck was significantly higher in athletes than in non-athletes [116]. In another study comparing triple jumpers and sedentary controls, it was shown that bone mass at the lumbar spine, proximal and distal tibia as well as in the femoral neck was significantly higher in the athletes [222]. However, not all types of exercise seem to be able to increase bone mass. For instance, in a study with skiers, cyclists, runners and weightlifters, it was shown that BMD was only significantly increased in weightlifters and runners compared to matched controls [220]. In another study involving individuals engaged in

weightlifting, running, cross-training, or recreational exercise, the results showed that the higher bone mass values were present in weightlifters while the lowest values were identified in the runners [211]. These results therefore suggest that not all exercise regimens have the potential to increase bone mass, and perhaps to increase bone mass it is necessary that exercise reaches a certain minimum of loading or frequency.

These is also evidence from several prospective studies showing that regular exercise is able to increase bone mass [382, 600]. For instance, young girls with ages between 9 and 10 years that were subjected to a physical exercise training program of 10-months duration evidenced significantly higher BMD increases in total body, lumbar spine, and femoral neck than age-matched sedentary controls [382]. Moreover, exercise training not only enhanced bone mineral accrual, as it also improved bone geometry and bone strength in growing boys that were subjected to a 20-months duration training program [333]. Although a large proportion of the increases in bone mass identified in this study were most likely due to age-related growth, these results demonstrate that exercise significantly enhanced bone mass gains. Moreover, in a study in which a group of adult men who trained during 9-months for a marathon race were compared with sedentary controls, it was shown that BMD at the end of the training period had increased significantly in the runners, but was unchanged in controls [600]. Moreover, increases in BMD were correlated significantly with the running distances performed [600], suggesting that exercise had a dose-dependent effect. These results also demonstrate that bone mass increases following exercise also occur in the adult and not just in the growing bones.

Despite there is evidence that genetic variation influences bone mass [439, 502] and the ability of bone tissue to adapt to mechanical loading [274, 439, 138], there is also compelling evidence showing that regular exercise is a major determinant of bone mass and structure, independently of the genotype. For instance, in a study with homozygotic twins [330] it was shown that bone mass and trabecular architecture were significantly increased in those that were physically active compared with those that were sedentary.

In all the previously mentioned studies, exercise was able to increase bone mass. However, these results alone make impossible to conclude if the effects of exercise on bone tissue were due to the whole physiological changes associated with exercise (heart rate, ventilator frequency, hormonal changes, temperature, etc), or if they were a consequence of the increased mechanical loading imposed to the bones. For instance, there is evidence that exercise is able to increase the circulating levels of growth factors [284, 167] which are known to increase bone mass [26]. Nevertheless, there is also a great body of evidence showing that the skeletal responses to exercise are site-specific [262, 294, 206, 205, 358] and that therefore are unlikely to result just from the action of systemic factors induced by exercise. For instance, in a previous study the differences between the dominant and non-dominant arm bone properties in a group of tennis and squash players were determined and compared to the differences between the dominant and non-dominant arm of a matched control group. It was shown that the between-arm differences were substantially higher in the players than in the controls [294], demonstrating therefore that the effects of exercise were localized, as the increases in bone mass occurred only in the arm that was subjected to mechanical stimulation.

2.2 Experimental studies on animals

Animal studies using *in vivo* well-controlled mechanical stimulation have also provided a wealth of information regarding the effects of exercise on bone formation and resorption [225, 462, 561]. The first experimental model of this kind was the *rat tibia four-point bending model* [559], being followed years later by the *rodent ulna axial loading model* [556], which is currently the most broadly employed *in vivo* mechanical stimulation protocol in experimental studies.

Regarding the application of controlled mechanical loading protocols, the work of Hillam and Skerry [225] was pivotal. In this study, the strains experienced by the rat ulna during normal deambulation were measured *in vivo* through the use of strain gauges. Considering the recorded values, the ulna of another group of rats was subjected to controlled axial loading [556] for 6 days. Histological analysis revealed

that bone formation was significantly increased while bone resorption was significantly decreased in the loaded ulna compared to the contra-lateral unloaded ulna. These results therefore support the concept that exercise, by increasing mechanical loading in bones, has the ability to increase bone formation and to decrease bone resorption in a site-specific way.

Experimental studies using the *in vivo* ulna loading model were also able to provide relevant findings regarding the characteristics of the loading regimen that are implicated in the osteogenic response. For instance, by comparing the osteogenic effect of static *vs* dynamic compressive loading regimens on the rat ulna, it was possible to verify that dynamic loading was able to increase significantly both periosteal and endocortical bone formation, while static loading regimens produced no osteogenic response [462]. In addition, by using the same loading model and by comparing the osteogenic effects of different loading frequencies with the same magnitude, it was demosntrated that higher loading frequencies were associated with higher bone formation rates [561].

2.3 Effects of unloading or disuse on bone

In opposition to regular exercise, extreme cases of disuse such as prolonged bed rest or microgravity are also able to demonstrate the importance that mechanical stimulation has for bone quality [304, 275, 299]. The effects of space flight on bone mass are well documented [495, 304, 93, 426, 275], and include loss of cortical and trabecular bone mass due to reduced bone formation and increased bone resorption, compromised trabecular microarchitecture and significantly reduced bone strength. For instance, in a recent study performed on astronauts that went on mission to the international space station, it was estimated that femoral neck strength decreased about 2.5% per month [275]. Studies using tail suspended rodents have also been used to mimic the effects of weightlessness on the skeleton [309, 506, 5, 396]. In general these studies also demonstrate that the absence of mechanical stimulation leads to a rapid bone loss. Similarly, the lack of mechanical stimulation due to prolonged bed rest has also been shown to induce significant bone mass losses [145, 299, 577, 310, 59,

626], with reduction in BMD reaching 0.9% per week [299]. Notoriously, there is also evidence that re-ambulation is able to restore the previous bone mass losses [299], despite in most cases losses are not fully reversed [310, 59].

3. MECHANISMS INVOLVED IN BONE TISSUE RESPONSE TO MECHANICAL LOADING

Although it is well established that bone tissue is responsive to mechanical stimulation and to the lack of it [225], the nature of the stimulus and the pathways involved in the conversion of mechanical signals into biochemical responses are still largely unknown. Nevertheless, there is evidence that several of the cells in bone, namely MSCs, osteoblasts, osteoclasts, endothelial cells and osteocytes, are able to be stimulated by mechanical signals influencing thereby bone turnover.

Several studies have shown evidence that MSCs are sensitive to mechanical stimulation, being the most noteworthy consequence of such stimulation a shift in their differentiation away from adipogenesis and towards osteoblastogenesis. For instance, *in vitro* studies show that oscillatory fluid flow applied to cultured bone marrow MSCs results in increased cell proliferation and messenger ribonucleic acid (mRNA) expression of osteocalcin and osteopontin, two proteins expressed by the mature osteoblast [320]. In another recent study [503], mechanical strain was also shown to inhibit in cultured MSCs the expression of adipocyte-related genes and to promote the expression of osteoblast-related genes, thereby shifting their differentiation fate towards the osteoblast lineage and away from the adipocyte lineage. This shifting effect has also been described in several *in vivo* studies, were application of low magnitude mechanical stimulus was able to increase MSCs number and differentiation towards the osteoblast lineage and away from the adipocyte lineage, resulting in decreased adipose tissue formation and increased formation of trabecular bone [472, 329].

The osteoblast has also been shown to respond to mechanical stimulation by increasing the secretion of signaling factors such as prostaglandin E2 (PGE2) and nitric oxide ($^{\bullet}NO$) [518, 172, 170] and by rearranging its cytoskeleton [424]. Mechanical stimulation was also suggested previously to directly target the osteoclast precursors, namely by inhibiting their differentiation, since bone marrow cultured cells subjected to mechanical strain had a significantly reduced osteoclast formation in opposition to unstimulated controls [474]. The decrease in osteoclast differentiation was shown

however in a later study by the same group [475] to be associated with a decrease in RANK-L expression. Therefore mechanical strain did not inhibited osteoclastogenesis directly; conversely this impairment was achieved through a reduction in RANK-L expression by osteoblasts.

The bone marrow and matrix are richly pierced by capillary vessels. Considering that the release of $^{\bullet}$NO from endothelial cells in response to fluid shear is well established [134] and that $^{\bullet}$NO is known to influence bone cells activity [477], some authors have suggested that vascular endothelial cells might also have a relevant role in bone adaptations to mechanical loading [117]. Nevertheless, whether $^{\bullet}$NO derives from bone cells or from the endothelium remains unclear, since $^{\bullet}$NO is also released by other bone cells, like osteoblasts [247] and osteocytes [622].

Due to the numerous lines of evidence suggesting that several types of cells in the bone are sensitive to mechanical stimulation, it is impossible to designate a unique cell type responsible for mechanotransduction. Perhaps all these cells contribute to the system homeostasis and therefore their mechanosensing role is at some extent redundant. Nevertheless, the morphology and ubiquitous presence of the osteocyte in the bone matrix, leaves it in the most favorable position for detecting and amplifying mechanical signals applied to the bone. Therefore, in recent years the osteocyte has been considered as the most relevant cell in mechanotransduction and on the orchestration of local bone remodeling in response to mechanical loading [66].

Osteocytes are ubiquitously present in bone tissue, interconnected with each other and to the bone surface by cellular projections that pierce through the bone matrix within canaliculi, while the cellular body remains housed in a lacuna [258]. These projections have gap-junctions at contact sites allowing direct cell-to-cell signaling between the osteocyte network [147], and between osteocytes and surface osteoblasts [611] placing therefore the osteocyte in a favorable position for orchestrating bone remodeling. The role of osteocytes in mechanotransduction is now well documented by several experimental studies showing that the osteocyte is responsive to different types of mechanical stimulation [545, 35]. There is evidence that mechanical stimulus lead to increases in osteocyte metabolism [516], gene

transcription [487] and to the secretion of signaling molecules [455, 16, 364, 464] that, by targeting other bone cells, ultimately lead to changes in bone turnover. For instance, results from a previous study shown that mechanical stimulation of co-cultured osteocytes and osteoclast-precursors leads to a significant decrease in osteoclast differentiation when compared to non stimulated cultures, which is a consequence of the reduction in RANK-L and an increase in OPG expression by the osteocytes [618]. Mechanical stimulation also appears to be able to inhibit osteoclast activation by preventing osteocyte apoptosis [438] while lack of stimulation is shown to increase osteocyte apoptosis [35]. For instance, a study by Aguirre et al. [13] demonstrates that mice subjected to unloading by tail suspension, have significantly increased osteocyte apoptosis, which is spatially and temporally followed by increases in osteoclast activation resulting in decreased bone mass and strength. It is also notorious that bone resorption following bone unloading is impaired if the osteocyte network is compromised [547], which highlights the role of the osteocyte network in the control of bone remodeling.

Osteocytes have also been shown to modulate osteoblast activity and MSCs commitment into the osteoblast lineage [218, 329]. However, the pivotal role of osteocytes in the regulation of bone formation, has in recent years, been shown to be associated with their ability to regulate the Wingless-int/beta-catenin (Wnt/β-catenin) signaling pathway. Wnts are secreted signaling glycoproteins [110, 624] that have been shown to be involved in bone tissue mechanotransduction [39]. Wnt/β-catenin cascade is initiated by the binding of Wnt to its transmembrane receptors [54, 542], which triggers glycogen synthase kinase-3 beta (GSK-3β) phosphorylation [625]. In a non-phosphorylated state, GSK-3β phosphorylates β-catenin leading to its ubiquitination and proteasomal degradation [2], which consequently reduces the levels of β-catenin in the cytoplasm. Wnt binding to its receptor however inhibits GSK-3β, leaving non-phosphorylated β-catenin free to translocate into the nucleus, activating thereby gene transcription [383]. Wnt/β-catenin signaling pathway has been shown to be associated with increases in bone mass through a number of mechanisms namely by increasing the proliferation of stem cells, increasing osteoblastogenesis,

inhibiting osteoblast and osteocyte apoptosis [298, 466, 195, 75], and by inhibiting osteoclast activation [192]. Recent findings also show that Wnt/β-catenin signaling is activated in osteoblasts in response to mechanical stimulation [306, 459, 491].

Wnt/β-catenin signaling is regulated to a great extent by sclerostin, a decoy protein secreted by osteocytes that prevents the interaction between Wnt and its receptors, thereby inhibiting the activation of the Wnt/β-catenin signaling pathway [442, 319]. For instance, administration of sclerostin to cultured osteoblasts inhibits their activity and stimulates their death by apoptosis [535], while inhibiting sclerostin leads to increases in bone mass [405, 413]. Importantly, there is evidence that osteocytes are able to regulate bone formation following mechanical stimulation by decreasing sclerostin expression [464, 324]. For instance, bones subjected to mechanical stimulation have a lower number of sclerostin positive osteocytes, while bones from suspended limbs have a significantly higher number of sclerostion positive osteocytes. Therefore, by regulating sclerostin expression, osteocytes are able to control bone turnover in accordance to their surrounding mechanical environment.

Considering therefore the evidence on the role of osteocytes in mechanotransduction, it is also possible that the progressive deterioration of the osteocyte network that typically occurs with age [385] may contribute to the lower responsiveness to mechanical stimulation previously identified in the elder [471].

3.1 Detection of the mechanical stimulus

Several studies demonstrate that the responses of bone tissue to mechanical loading are influenced by mechanical strain magnitude [473], loading frequency [228] and intermittency [463, 525]. Nevertheless, how these external signals are sensed, translated and conveyed between the osteocyte networks still remains largely unknown, with evidence suggesting the involvement of several pathways [592, 122, 425, 43, 268, 485, 599, 608, 337, 263].

As bone is not a rigid entity, mechanical forces applied to it are at some extent dissipated by elastic deformation [182] witch consequently induces deformation of the osteocyte membrane and/or cytoskeleton and causes a flow of water and solutes

through the canalicular network [524]. Although whole bone strains experienced *in vivo* are of very small magnitude, it has been proposed that the canalicular network has the ability to amplify these strains [592, 212] creating a useful stimulus. Ultrastructural findings [619] evidence the presence of the basic elements involved in the amplification models, namely the existence of a proteoglycan rich pericellular matrix surrounding the osteocyte processes witch increases fluid drag forces and shear stresses, projections that anchor the osteocyte processes to the canaliculi as well as the presence of elements of the cytoskeleton within the cell processes. This amplification model [617] proposes that fluid drag on the pericellular matrix is transmitted to the projections surrounding the osteocyte process, causing them to deform which results in the generation of a tensile force that is transmitted to the osteocyte cytoskeleton. These findings form the basis for the three main proposed mechanisms for mechanosensing, which are shear stress, membrane deformation and cilia presence.

Fluid flow through the canalicular system is believed to be one of the most important stimuli following mechanical loading in bone [592, 122] as different types of flows, such as laminar [20], pulsatile [545] or oscillatory [417] flow, have been successfully used *in vitro* to induce osteocyte stimulation. Nevertheless, direct evidence of the existence of canalicular fluid flow *in vivo* is still limited [587]. In order to detect and respond to mechanical signals, cells are required to be either physically attached to the ECM or to have structures that are able to detect changes in an intermediary such as in the extracellular fluid flow [18]. Modification of membrane potential through channels is one of the most widespread biological strategy for regulating cell behavior and there is evidence that mechanical stimulation in osteoblasts [133, 215], and osteocytes [451], leads to the activation of mechanosensite [451] and voltage-sensitive ion channels [133, 317, 215] thereby resulting in the activation of intracellular signaling pathways.

Integrins are transmembrane proteins involved in the attachment of the cell cytoskeleton to its surrounding ECM and in signal transduction through the activation of protein kinases that are associated with the intracellular domain of the integrin. As

there is evidence of the presence of integrins on the osteocyte [368] and osteoblast membranes [313, 598] it is believed that integrins also have an active role in the detection of mechanical loading in bone [425, 43, 268, 485].

More recently, a mechanism for detecting fluid flow based on the presence of cilia, similar to what is observed in renal epithelial cells [325, 443, 389], was also been proposed as a mechanosensing mechanism in bone cells [599, 608, 337].

As there are several lines of evidence supporting each one of the proposed mechanisms, it is most likely that a certain redundancy exists and that no single receptor or mechanosensing pathway is responsible for detecting mechanical stimulus in bone, especially if we consider that mechanical stimulation is able to activate several signaling pathways in bone cells [263].

3.2 Mechanotransduction

Mechanical signals sensed by bone cells eventually activate intracellular signaling pathways that ultimately alter cell function, gene expression and induce the release of second messengers. This process is designated by mechanotransduction. Owing to the existence of several mechanosensing strategies, there is evidence that several signaling pathways are involved in mechanotransduction in bone cells.

G-protein coupled receptors (GPCR) are a large group of receptors that are able to mediate cellular responses to several different stimulus and studies on endothelial cells have shown that GPCR are activated by fluid shear stress [98]. The role of GPCR signaling in bone cells mechanotransduction has also been documented [454, 30, 331].

Mechanical stimulation on bone cells also leads to an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) [231], which is shown to depend both on the entrance of extracellular calcium through voltage-dependent calcium channels as well as on the mobilization of intracellular calcium stores [231, 583, 616, 102]. For instance, *in vivo* studies show that blocking calcium channels leads to a decrease in bone formation following mechanical stimulation [317]. The activation of osterix, an essential transcription factor controlling osteoblastogenesis, is also controlled by $[Ca^{2+}]_i$ increase. Calmodulin activation by calcium leads to activation of calcineurin, with in

turn activates nuclear factor of activated T-cells-2 (NFAT2). Activated NFAT2 assembles then with Osterix and promotes its nuclear migration where it up regulates the transcription of osteoblast specific genes such as type I collagen [289, 533].

Mitogen activated protein kinases (MAPKs) are involved in several intracellular signaling pathways and there is evidence suggesting that they are also involved in bone cells response following mechanical loading [616, 477, 476, 261]. For instance, in has been previously shown that mechanical strain applied to cultured osteoblasts increases their proliferation, which in turn is prevented by inhibiting MAPK signaling [72].

Nitric oxide is a highly soluble gas with an important role as an intra and intercellular messenger, and it has also been shown to be implicated in mechanotransduction in bone cells [449, 162].

3.3 Propagation of biochemical signals between bone cells

It is believed that osteocytes are able to communicate between then and with other cells through different ways. One of the most broadly studied and for which there is more evidence is direct communication through gap junctions. There is evidence of the presence of connexin43 (Cx43), a gap-junction protein, in osteoclasts [494], osteoblasts [113] and osteocytes [204]. The importance of gap junctions for normal bone function is suggested in several studies where experimental animal or humans with Cx43 mutations exhibit several ossification disorders [311, 91, 509]. Moreover, gap junctions have been shown to play a role in osteoblast differentiation and proliferation following mechanical stimulation [197, 321, 549, 630, 104, 436]. Additionally, there is evidence that mechanical stimulation increases Cx43 expression in osteocytes [19].

Prostaglandins are signaling molecules derived from fatty acids that are involved in cell signaling between a great diversity of cells. Several studies have provided evidence suggesting that PGE2 is involved in the communication between bone cells, namely between osteocytes and osteoblasts, following mechanical stimulation [111, 331], and that cultured bone cells mechanically stimulated have an increase in PGE2 production

[489, 45, 498]. Moreover, administration of PGE2 is able to increase bone formation in experimental animals [240], while inhibition of cyclooxygenase-2 (COX-2) inhibits bone formation induced by mechanical stimulation [112, 170, 482, 291].

Although adenosine triphosphate (ATP) main role is to act as an energy carrier, it can also mediate cell signaling by binding to purinergic receptors which are either ligand-dependent ion channels (P2X receptor) or GPCR (P2Y) and studies on cultured osteoblasts have shown that ATP is also involved in cell signaling in bone following mechanical stimulation [615, 121, 404, 187].

Despite the broad evidence supporting gap-junctional based communication importance between bone cells, it is also known that mechanically stimulated osteocytes are able to release $^{\bullet}NO$, which has the ability to rapidly cross the cell membrane and to diffuse to neighboring cells [572] independently of gap junctions.

4. VARIATIONS IN BONE TISSUE RESPONSIVENESS TO EXERCISE WITH AGE AND ESTROGEN

According to the mechanostat theory [176] bone mass is regulated in a way to ensure that bone strain is kept within a certain physiological range. Therefore, when loads imposed to the skeleton lead to excessive strain, there is a compensatory increase in bone mass in order to reduce bone strain to within the safety range. However, this theoretical model by witch bone mass adapts to mechanical loading seems to fail in the elder and after estrogen loss [178]. Together with the gradual decline in bone mass seen with ageing and after the menopause, the clear anabolic effects of mechanical loading seen in adolescents and young adults apparently do not occur in ageing adults [562, 471]. This observation has lead to the belief that bone tissue responsiveness to mechanical loading may be reduced with ageing and low estrogen levels [177].

4.1 Estrogen Receptors are involved in the response of bone cells to mechanical loading

There is some evidence suggesting that a possible reduction in bone tissue responsiveness to mechanical stimulation associated with low estrogen levels might be linked with abnormalities involving ER signaling pathways as there is evidence that ER are involved in mechanotransduction in bone cells.

The ER are ligand-inducible transcription factors that after interacting with estrogen, bind to estrogen response elements activating thereby gene transcription in the cell [419, 123, 300]. ER are expressed in osteoblasts [137, 158], osteocytes [621], MSCs [403] as well as in osteoclasts and osteoclast precursors [407], and they mediate the stimulatory action of estrogen on bone formation [363]. In addition to their classical ligand-induced activation, ER have two transcriptional activation domains [362] that have been demonstrated to allow ligand-independent activation following phosphorylation [154], namely via MAPK [266] and protein kinase A (PKA) [154] pathways, which are two intermediates involved in mechanotransduction in bone cells

[72, 581]. Studies on cultured osteoblasts demonstrate that, soon after being stimulated with estrogen or being subjected to mechanical stimulation, there is an increase in the expression of activated ER [242]. Moreover, the activation of ER by strain and estrogen was demonstrated to be associated with the activation of MAPK and PKA signaling pathways, since ER activation was prevented in both cases by the introduction of MAPK and PKA inhibitors [242]. In other studies it was also demonstrated that mechanical strain and estrogen were both able to stimulate the proliferation of cultured osteoblasts [620, 105]. Moreover, osteoblast proliferation following mechanical strain was shown to be greater in cells expressing higher amounts of ER and was blocked by the presence of ER antagonists [620]. Interestingly, the bone mass increase following mechanical stimulation was also shown to be significantly lower in mice lacking ER than in wildtype mice [314]. Moreover, following mechanical stimulation, cultured osteoblasts lacking ER showed no signs of proliferation, whereas in normal osteoblasts there was a significant increase in proliferation [314, 243]. These results therefore suggest that ER are required for the proliferation of osteoblasts following mechanical stimulation, and that a reduction in the expression of ER might impair bone mass increases following mechanical stimulation.

There is also evidence suggesting that ER are involved in Wnt/β-catenin signaling pathway [28, 296], which is an important signaling pathway mediating the response of bone cell to mechanical stimulation [459]. The key event in Wnt/β-catenin signaling is the translocation of β-catenin into the nucleus where it leads to the up regulation of gene transcription [383]. However, β-catenin apparently requires association with other transcription factors to pass through the nuclear membrane pore as it is shown to lack the required nuclear recognition sequences [223, 213]. Interestingly, there is evidence from both cultured osteoblasts as well as *in vivo* studies, that inhibition of ER significantly impairs the accumulation of active β-catenin in the nucleus and reduces the expression of Wnt/β-catenin related genes in response to mechanical strain [28]. These results therefore suggest that ER are somewhat required for the occurrence of a

mechanical strain induced response in osteoblasts through the Wnt/β-catenin signaling.

In addition to its effects on osteoblast [132, 493, 245] and osteoclast activity [69, 226, 481, 257, 229, 388], mechanical stimulation has also been shown, in *in vitro* studies, to promote osteocyte viability, which is considered to be a key aspect of bone quality [547, 448]. Interestingly, mechanical stimulation [438] and estrogen [555] have been both shown to promote osteocyte survival through the activation of extracellular signal-regulated kinases (ERK), which suggests that they share common intracellular pathways. Moreover, inhibition of ER was shown to prevent the mechanical strain induced activation of ERK in cultured osteoblasts and osteocytes, and to abrogate the anti-apoptotic effects of mechanical stimulation over osteocytes and osteoblasts [12]. These findings therefore suggest that ER are involved in the signaling pathways through which mechanical strain prevents osteocyte apoptosis. Thus, abnormalities in ER expression following estrogen loss might reduce the effects of mechanical stimulation on the promotion of osteocyte viability.

Notoriously, there is evidence suggesting that ER expression is regulated by circulating estrogen levels [51, 8], and that estrogen administration significantly increases ER expression [536]. For instance, by comparing the ER expression in the osteocytes from OVX and SHAM animal´s tibiae, Zaman and colleagues [621] showed that ER expression was significantly lower in OVX animals. Together these findings suggest that the cessation of estrogen production leads to a reduction in the availability of ER in osteoblasts. Hence, this reduction could be associated with the impairment of mechanical strain signaling therefore leading to diminished bone responsiveness to mechanical loading following estrogen loss.

4.2 Possible effects of age and estrogen levels on bone tissue responsiveness to mechanical loading

Despite that both mechanical stimulation and estrogen have well documented effects on bone cells and the evidence that they share some common intracellular signaling pathways, whether they act synergistically or not in modulating bone cells

activity remains largely unknown. There is evidence from *in vitro* as well as from animal and humans studies, suggesting that estrogen is able to increase [610, 249, 36, 293], to decrease [492, 235] or even that it has no influence [415] on the osteogenic response of bone cells following mechanical stimulation.

In a very recent study with the objective of determining if estrogen influences the response of bone cells to mechanical stimulation [610] it was shown that both estrogen as well as shear stress were able to increase the expression of target genes in cultured osteoblasts. However, estrogen was able to significantly enhance the effects of shear stress. The blocking of ER in turn prevented the enhanced response provided by shear stress and estrogen in concert, but did not prevented shear stress from activating the target genes. Interestingly, estrogen has also been shown to increase the expression of membrane integrins on osteoblasts. Integrins are known to have an important role in mechanotransduction [323] and to be involved in osteoblast differentiation and commitment [200]. In this study [610], the estrogen enhancement of shear stress induced gene activation was shown to be associated with the increase in integrin expression. Similar results were also demonstrated in a previous study [249], in which stimulation of cultured human osteoblasts by fluid flow was able to increase the expression of target genes. Estrogen in association with fluid flow was able to enhance the expression of some but not all of the genes activated by fluid flow. These findings therefore suggest that although estrogen was able to increase the osteoblast responsiveness to mechanical stimulation, lack of estrogen did not impaired the response of bone cells. If this is so, mechanical strain following estrogen loss need to be of higher magnitude than in during estrogen presence in order to produce an effective anabolic response in bone. These conclusions are in agreement with the findings of another study [36], that describes the response to fluid flow of bone cells from osteoporotic women that were cultured in the presence or absence of estrogen. In this study, both estrogen and fluid flow were able to stimulate PGE2 and $^{\bullet}NO$ expression independently. However, the simultaneous stimulation by estrogen and fluid flow promoted an increased response compared to the application or either

stimuli alone, suggesting that the actions of mechanical stimulation and estrogen are independent but additive.

The apparently additive effect of estrogen and mechanical stimulation appears to be associated with their independent action over whole bone structure, suggesting the existence of *in vivo* regional differences in bone response to loading and estrogen, that are unable to be fully apretiated in cell culture studies. By analyzing microarchitecture, bone geometry and strength of bones from OVX and SHAM animals that were either loaded or cast immobilized, Järvinen and colleagues [415] verified that the overall effects of loading were identical between animals that lacked or not estrogen. Noteworthy however was that the effects of estrogen and loading were anatomically distinct. While loading induced increases in periosteal apposition with resorption occurring mainly in the endocortical surface, estrogen was associated with both periosteal and endocortical bone apposition. The regional differences of estrogen and loading induced bone adaptation are also evidenced in two studies where estrogen was shown to inhibit periosteal bone formation and enhance endocortical bone apposition [281] while in another study the mechanical loading produced mostly increases in periosteal bone formation in the rat cortical bone [288]. Interestingly, the pattern of bone formation following exercise has also been shown to be different according to the pubertal state. Bass et al. [42] verified that while in pre-pubertal tennis players, who have low estrogen levels, exercise induced mostly an increase in periosteal expansion in the playing arm, in post-pubertal girls who have comparatively higher levels of estrogen, exercise enhanced mostly endocortical bone formation. It is therefore possible that when associated with exercise, estrogen somewhat inhibits the periosteal bone formation that would be archived by exercise stimulation alone but simultaneously enhances bone formation in the encortical region more than it would be achieved by exercise alone. In order to test the hypothesis that estrogen reduces the exercise induced periosteal expansion while enhances endocortical bone formation, Saxon and Turner [492] stimulated by mechanical loading the ulna of male rats supplemented with either estrogen or vehicle. Loading resulted in significantly higher increases in cortical bone area and bone mass in animals that were not

receiving estrogen. Moreover, periosteal bone formation rate was 2-fold lower in animals that received estrogen, while no differences were identified in the endocortical region.

Besides the regional differences in bone response to loading and estrogen, the temporal pattern of estrogen and mechanical stimulation also appears to influence the bone adaptive response. This is evidenced in a study where *in vivo* estrogen administration suppressed while estrogen loss enhanced the early anabolic effects of mechanical stimulation on bone [235]. In this study, a single episode of cyclic loading of the vertebra resulted in a significant increase in bone formation that was suppressed by the administration of estrogen before or after the load. Interestingly, when estrogen was given 3 days after mechanical stimulation, a significant enhancement of the anabolic response was identified, suggesting that estrogen was only able to increase the activity of previously stimulated bone cells by loading. However, in another study, the administration of estrogen to OVX animals once a week was unable to enhance the exercise induced increases in BMD and bone mineral content (BMC), showing that exercise alone was able to increase bone mass in OVX animals [557].

Results from studies performed on post-menopausal woman are also inconsistent regarding the influence of hormone replacement therapy (HRT) in enhancing the effects of mechanical loading. Kohrt et al. [293] verified that both lumbar spine and proximal femur BMD were significantly increased in response to exercise or HRT alone in the elder women. However, when combined they resulted in additive effects in some skeletal sites but not in others. Identical results are also described by Milliken et al. [375], in which the combination of exercise and HRT produced higher increases in BMD of postmenopausal woman than either one alone. As it was previously mentioned, variations in the regional expression of different ER isoforms as well as variations according to osteoblast differentiation stage [68, 406] might originate at least some of the different and site specific results described regarding the effects of exercise and HRT alone or in combination. The documented evidence regarding the interaction between estrogen and mechanical stimulation shows then a very complex

picture with the involvement of several variables that seem to be time and place dependent.

Studies using different exercise regimens as well as controlled bone loading have also reported conflicting findings regarding the variation of bone tissue responsiveness to mechanical loading with age, with studies reporting either reduced responsiveness in aged animals, no difference between young and old animals or even enhanced responsiveness in aged animals.

There is some evidence that the anabolic response of bone to loading varies with age. Results from previous studies suggest that higher mechanical loading thresholds are necessary for promoting bone formation in aged compared to young rats [562] and that the ulna from young turkeys is substantially more responsive to mechanical stimulation than the ulna from 3-years-old turkey [471]. Nevertheless, there are also several studies suggesting that bone from aged animals is as responsive as bone from adult animals when submitted to exercise and that there is no loss of mechanosensitivity with age. For instance, a 14-week treadmill running program on male and female mice with either 47 or 75 weeks of age resulted in overall increases in bone mass and strength that were higher in the older group, particularly in female animals [316]. Similarly, in the rat with back-pack model, neither young (4 months) or adult (12 months) animals were significantly influenced by exercise, while in the older (22 months) animals there was a decrease in marrow area and in trabecular bone separation [83]. In another very recent study [80] in which two groups of male mice with 7 and 22 month of age were subjected to tibial axial compression, it was demonstrated that the response to loading in aged mice was significantly higher compared to young mice. Moreover, mechanical loading was shown to significantly reduce bone resorption in old mice.

One of the possible reasons for the contradictory findings between several studies is that age might have different effects on bone cells, leading to different conclusions whether the outcomes are considered at a cell or tissue level. A study by Klein-Nulend [286] shows that the response of cultured bone cells to fluid flow varies according with the donor age. While cell proliferation significantly decreased according to donor age,

basal ALP activity and PGE2 expression increased with donor age following mechanical stimulation. This suggests therefore that probably there is no loss of responsiveness with age. What happens is that the response following mechanical stimulation most likely suffers a gradual change leading however to similar whole bone effects after *in vivo* stimulation since increased cell activity compensates for reduced cell proliferation.

5. EXERCISE IN THE PREVENTION AND TREATMENT OF OSTEOPOROSIS

5.1 Peak bone mass

During childhood and adolescence the skeleton grows progressively, with individual bones increasing not only in length but also in cross sectional area due to a gradual periosteal expansion. This process is designated bone modeling [428]. The gradual increase in bone mass observed during growth reaches a peak at about 20 years of age [355], stabilizing thereafter. This point of maximal bone mass accumulation at the end of the growth period is designated as peak bone mass, and it is determined by several factors, such as genetic background, hormonal status, nutrition and physical activity [107, 216].

Considering therefore the existence of a period in life at which bone mass reaches a maximum and that afterwards it declines progressively until reaching dangerously low levels decades later, several authors have argued that osteoporosis could be the late outcome of a low peak bone mass during growth [165, 354, 107]. Consequently, interventions such as exercise with the potential of enhancing the peak bone mass during growth were considered to be an effective strategy to prevent or delay the onset of osteoporosis in the adult [41, 367]. In fact there are several studies demonstrating that exercise is able to enhance peak bone mass in the growing child and adolescent [77, 382, 221, 179].

For instance, a group of 10-year boys that were engaged in an 8-month regular exercise training program, demonstrated 2-fold higher BMD increases than age-matched controls at several skeletal sites [77]. In another study with a group of 10-year girls enrolled in a exercise training program, it was shown that at the end of 10-months their BMD was significantly higher than the BMD in a group of age-matched controls that were sedentary, despite the absence of initial differences between the two groups [382]. Several experimental studies with growing animals submitted to exercise training protocols have also demonstrated that bones from these animals have significantly higher increases in cortical bone thickness, cortical bone cross-sectional area and ultimate strength than bones of non-exercising counterparts [490, 171, 237, 357].

Nevertheless, the bone mass of the elder and post-menopausal women depends not only on the amount of bone acquired during growth, i.e. peak bone mass, as also on the rate of the progressive bone loss during adulthood. So, for a higher peak bone mass to be able to grant some advantage at old age it should be reflected as a higher bone mass later in life. In a retrospective study that compared BMD of a group of 50-years old retired ballet dancers with that of a group of age matched controls that were sedentary during youth, it was shown that former ballet dancers had a significantly higher BMD than the controls. Moreover, higher differences in BMD between groups were associated with higher self reported training hours [277]. In another interesting study [41] in which the bone mass of a group of 10-year old female gymnasts was assayed and compared to that of sedentary controls, it was demonstrated that gymnasts had a significantly higher BMD than controls. Moreover, during a 12-month duration exercise training program, BMD increases were 85% higher in the group of gymnasts than in controls, demonstrating therefore that exercise was able to significantly enhance bone mass accrual in the young, boosting thereby their peak bone mass. Notoriously, in this same study, the BMD of a group of retired 25-years old gymnasts was also determined, and it was shown that their BMD was significantly higher than that of controls, suggesting therefore that the benefits of increasing peak bone mass were maintained into adulthood.

Nevertheless, despite that the benefits of increasing peak bone mass might be detected in the adult, several lines of evidence suggest that exercise-induced bone gains achieved during growth are not preserved in the elder [264, 414, 602]. To investigate if increasing bone mass through exercise in the growing rat would have a meaningful effect on the bone mass at old age, Pajamaki et al [414] subjected a group of young rats to an exercise training protocol of 14 weeks of duration. At the end of the training protocol the animals were put back on normal cage activity and were scarified following 14, 28 or 42 weeks. Interestingly, despite bone mass was significantly higher immediately after the training protocol in exercised animals compared to controls, at the end of the 42 weeks of deconditioning there were no differences between previously exercised animals and controls that were kept in

normal cage activity through life. In another pivotal study with former soccer players and age matched controls [264], it was shown that despite exercise during growth promoted an increase in peak BMD, after retiring from practice the soccer players experienced an accelerated bone loss, resulting that at 60 years of age there were no differences between former athletes and controls regarding BMD and, most importantly, fracture rate. Therefore, these important findings demonstrate that reaching a high peak bone mass alone is not enough to prevent osteoporosis or increased risk of fracture at old age since exercise-induced bone benefits are eventually lost if exercise is not maintained. Therefore, the maintenance of a regular physical activity through life is most probably necessary to protect the skeleton from accelerated bone loss and thereby to prevent fractures.

5.2 Exercise programs for increasing bone mass

Despite the evidence provided by studies with animals [238, 81] and humans [600] that adult bone still preserves its responsiveness to mechanical loading and exercise training, conclusions between different studies have been somewhat conflicting. In the last 15 years several meta-analysis and systematic reviews have analyzed the results of studies in which the effects of exercise on bone mass and fracture risk were assessed [271, 272, 349-351, 416, 584, 604, 273]. The main question that these large studies were tiring to answer was if post-menopausal woman were able to increase or maintain their BMD by performing regular physical activity. The answers though have been inconsistent.

One of the first meta-analysis performed with the objective of determining the effects of physical activity programs on bone mass of healthy postmenopausal women, was performed by Bérard and colleagues [52]. Only prospective controlled intervention trials with healthy postmenopausal women over 50 years of age were included, regardless of the type of exercise program or if the exercises were targeted or not to a specific body region. Eighteen studies were analyzed, and it was concluded that physical activity had a significant effect on BMD at the lumbar spine, but no effect what so ever at the forearm or femoral neck. A year later another meta-analysis

examining the effects of just aerobic exercise training on the lumbar vertebra BMD of postmenopausal women concluded that BMD was significantly higher in exercised women compared to sedentary controls. However, the higher BMD observed was mostly caused by the significant decreases in BMD in non-exercising women rather than being the result of true increases in the exercising group [271]. In the same year, these authors presented another meta-analysis [272], this time analyzing the effects of aerobic exercise on the femoral neck BMD, in which it was concluded that aerobic training was not only able to prevent decreases in BMD as it was also able to produce significant increases, contrasting therefore with previous analyses performed by others [52].

In another study analyzing randomized controlled trials about the effects of impact and non-impact exercise training programs on the lumbar vertebra and femoral neck BMD of post-menopausal women, it was concluded that both exercise regimens were able to increase BMD significantly at both skeletal sites [584]. However, results from meta-analysis on studies about the effects of programs involving walking-alone exercises concluded that significant increases were observed in lumbar vertebra BMD but not in the femoral neck [416]. Interestingly, a few years later another meta-analysis was performed analyzing previously published data on the effects of walking-alone exercises on lumbar spine and femoral neck BMD and concluded that exercise had no effects what so ever on the spine whilst significant improvements in the femoral neck were observed. Moreover, in another more recent meta-analysis [273] in which controlled trials that assessed the effects of exercise training on femoral neck BMD were analyzed, it was concluded that exercise was unable to significantly increase BMD at any skeletal site in post-menopausal women. Therefore, the main outcome of these studies is the inconsistency of previous findings regarding the effects of exercise on preventing the loss or in promoting the increase in BMD in post-menopausal women.

Notoriously, all the published data analyzed in these meta-analyses included only studies in which the primary outcome used to evaluate the effects of exercise on bone was BMD changes assayed by DXA. Regardless of its value for diagnosing osteoporosis,

BMD increases correlate poorly with fracture risk reduction following treatment [94], which suggests that its usefulness as a surrogate for detecting treatment effectiveness may be low [500]. Bone mass assessment with DXA has a number of limitations due to its two-dimensional nature as well as due to its low spatial resolution [236]. These limitations do not allow DXA to identify changes in trabecular or cortical architecture, changes in bone geometry as well as changes in bone tissue properties that can largely and independently influence bone strength. Consequently, assessing treatment effectiveness by monitoring just BMD changes is insufficient and likely misleading and would instead be best achieved by monitoring a broad set of bone properties that might change with treatment reducing thereby fracture risk. It is important to mention again that BMD is not a synonymous of bone strength, and that bone strength depends on several material and structural properties [501]. Moreover changes in these properties are not necessarily captured by a shift in BMD during treatment [136] as we see for instance in cases were fracture risk is reduced without increases in BMD [488] and in cases where large increases in BMD do not reduce fracture risk [208].

Despite the inexistence of randomized control trials using fracture rate as a primary outcome for determining the effectiveness of exercise programs in the prevention of osteoporosis, some prospective studies have been published providing data about fracture occurrence and habitual physical activity levels of the participants. An interesting recent meta-analysis has analyzed results from these studies and has concluded that moderate-to-vigorous physical activity was associated with a reduction of about 45% and 38% fracture risk in men and women above 40-years of age, respectively. These results therefore suggest that regular physical activity significantly reduces fracture occurrence in late adults and elder individuals, and that the inconclusive and quite reduced effects of exercise on the BMD of these individuals is not likely to be the main responsible for that reduction in fracture rate.

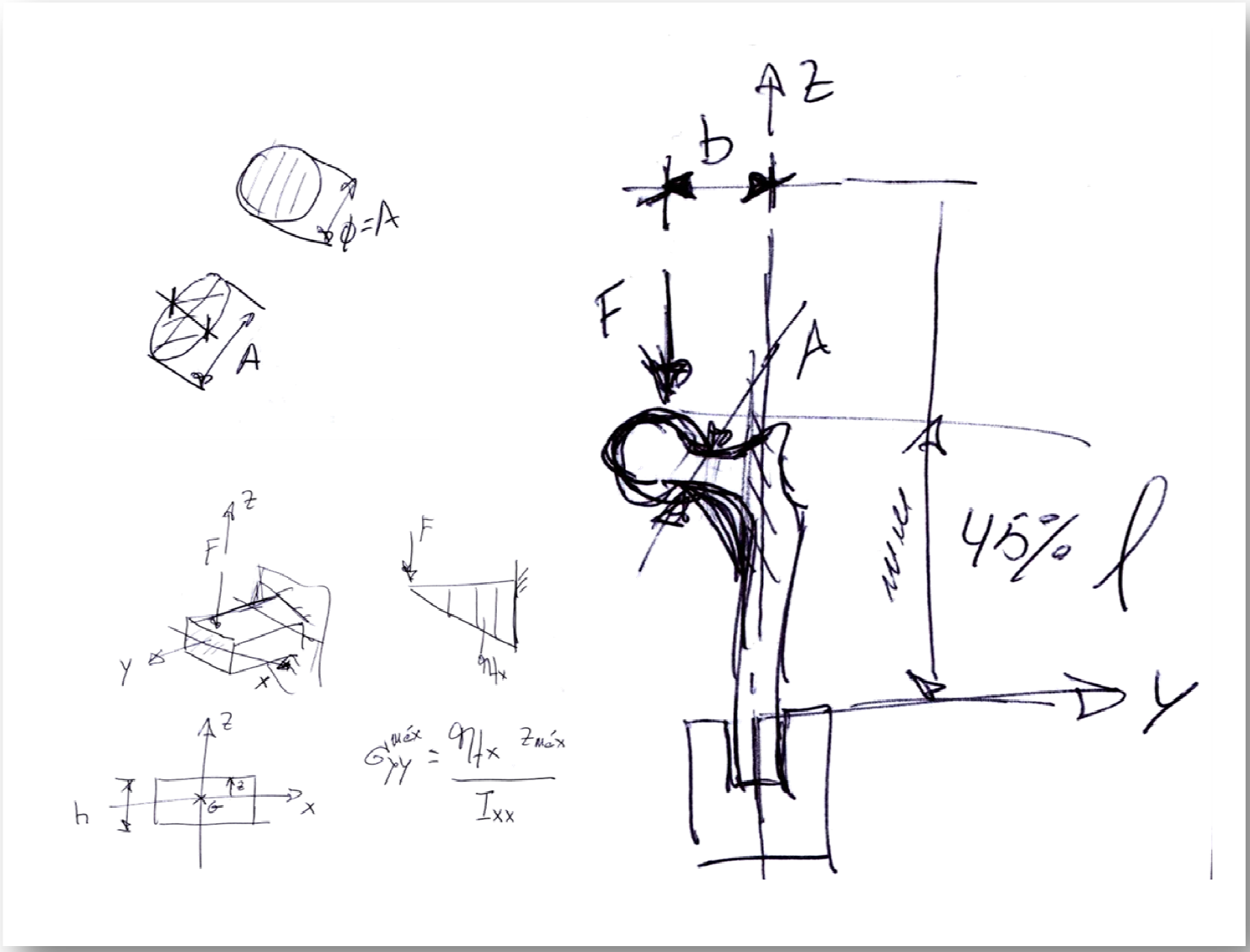
Nevertheless, as exercise also increases skeletal muscle mass [539] and improves balance [507], one might argue that the reduction in fracture occurrence reported in this study might not reflect true bone strength gains but rather a reduction in the number of falls witch consequently would result in a reduction of the number of

fractures. However, data from another interesting study suggests that this reduction does not result only from a reduction in the number of falls. In a randomized controlled trial Adami and collegues [7] applied an exercise program to a group of 250 postmenopausal women for 6 months. All participants were assayed by DXA and peripheral quantitative computed tomography (pQCT) at baseline and after the training program. DXA results showed that the exercise program had no effects on BMD as there were no significant differences between exercised women and controls. Interestingly, pQCT revealed that exercise training increased the radius cross sectional area significantly. Moreover, due to its ability to distinguish between cortical and trabecular bone, pQCT revealed a significant increase in cortical bone BMD and a decrease in trabecular bone BMD, suggesting therefore that there was a redistribution in bone mass away from the bone neutral axis, witch from a mechanical standpoint significantly increases its strength. This study therefore provides two important findings. First, that exercise was able to induce differences in bone geometry and mineralization degree that are consistent with an increase in bone strength, and second, that DXA was unable to identify those alterations. In another study [237] performed on experimental animals that were submitted to an exercise protocol during 9-weeks, it was showed that exercise induced a significant increase in cortical bone thickness and cross-sectional area in the animals resulting in a significantly increased bending and compression strength of the femoral shaft and neck, respectively. Quite notorious however was that no differences were detected between groups regarding DXA derived BMD measurements.

Therefore, exercise interventions seem to be able to increase bone strength and to reduce fracture risk by reorganizing bone mass distribution, an effect that seems to have been greatly underestimated by DXA derived BMD measurements.

CHAPTER III

Experimental Studies



Study 1

Fonseca H, Powers SK, Moreira-Gonçalves D, Santos A, Mota MP, Duarte JA. **Physical inactivity is a major contributor to ovariectomy induced muscle loss**. (*Submitted* to Int J Sports Med)

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Menopause related sarcopenia

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Title

Physical inactivity is a major contributor to ovariectomy-induced muscle loss

Running title

Menopause related sarcopenia

Abstract

Introduction: Since the mechanism(s) underlying menopause-related sarcopenia remain unknown we aimed to investigate the role of physical inactivity in its etiology. *Methods*: Ovariectomized and sham-operated rats were allocated into two experimental groups: 1) sedentary-standard housing; and 2) exercise-housed with running wheel. After a 9-month experimental period, soleus muscle structure and biochemical properties were analyzed. *Results*: No differences existed in muscle fiber size or ultrastructure between sedentary sham and ovariectomized animals housed in standard conditions. In the exercise groups, average daily running distance was 10-fold less in ovariectomized compared to sham-animals. Further, in exercised animals, soleus fiber size was smaller in ovariectomized compared to sham-animals. Nonetheless, compared to both sedentary groups, muscle fiber size was larger in the exercised ovariectomized animals. *Discussion*: Our results indicate that ovariectomy-induced sarcopenia is not due to the loss of ovarian hormones per se, but is largely due to physical inactivity.

Keywords

Voluntary running; Menopause; Sedentary behavior; Sarcopenia; Estrogen

Introduction

Menopause is a milestone in women's health, as the loss of regular estrogen production is typically followed by an increased occurrence of several detrimental musculoskeletal and cardiovascular changes. Among those, decreases in skeletal muscle mass and function are major outcomes of menopause and they are thought to be directly linked to hypoestrogenemia [9]. Skeletal muscle in several animal species, including humans [17] express estrogen receptors (ER) making muscle fibers a target tissue of estrogen action. A decrease in skeletal muscle mass and strength is a well-documented outcome of menopause. [9] This menopause-related loss of skeletal muscle mass and strength can be prevented, at least in part, with estrogen replacement therapy [30]. Ovariectomy (OVX) in mice also leads to significant decreases in locomotor muscle strength [21] which can be successfully restored following estrogen treatment [20]. Several mechanisms have been proposed to explain the loss of skeletal muscle mass and strength induced by estrogen deficiency [2-6,16,25,26,32,35,37]. For example, changes in mice soleus muscle contractile properties following OVX are shown to be associated with a significant shift in muscle fiber composition from fast to slow myosin heavy chain (MyHC) isoforms [16]. Ovariectomy is also known to induce alterations on the extracellular matrix of the rat heart by promoting an increase in type I collagen expression and a decrease in matrix metalloproteinase 2 expression, both of which are prevented by estrogen supplementation [38].

Furthermore, low estrogen levels could also have a negative impact on skeletal muscle energy metabolism as mitochondria are an estrogen sensitive organelle [5] as estrogen administration to OVX animals enhances respiratory chain activity [25]. Interestingly, some of the impairment within skeletal muscle of OVX animals has been shown to be prevented by regular bouts of muscular exercise [16]. This observation raises the possibility that the impact of estrogen deficiency on skeletal muscle is not due to estrogen deficiency alone. In this regard, evidence suggests that estrogen plays an important role in voluntary physical activity because low estrogen levels is associated with physical inactivity [11]. Indeed, OVX rats have been reported to run significantly less when compared to age matched intact controls [34]. Furthermore, estrogen replacement can effectively reverse OVX induced physical inactivity in mice in a dose-dependent fashion [8,11]. Collectively, these data suggest that low estrogen levels are associated with a decrease in voluntary physical activity. It follows that the decreases in skeletal muscle mass and strength following menopause could be linked to reduced physical activity (i.e., muscle disuse) and therefore, may not be directly linked to the

effect of low estrogen levels on skeletal muscle fibers *per se*. To investigate this important issue we tested the hypothesis that physical inactivity is a major contributor to menopause related sarcopenia. Specifically, we determined if the loss of ovarian function has a direct effect on skeletal muscle structural and biochemical properties or if the changes in skeletal muscle following loss of ovarian function largely reflect muscle disuse due to decreases in voluntary motor activity.

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Materials and methods

Overview of experimental approach: To determine the role that estrogen plays in skeletal muscle structure and size as well as the role of estrogen in promoting physical activity, we performed the following experiment. Our experimental approach was to study ovariectomized and sham operated female rats that were housed with access to running wheels or housed in standard, sedentary conditions. We recorded the levels of physical activity in all animals during a 9-month period and at sacrifice we evaluated the structural and biochemical properties of the soleus muscle.

Study design: All procedures involving animal care and sacrifice were approved by the local ethics committee (University of Porto) and meet the ethical standards of the journal [13]. Following one week of quarantine after arrival, 25 nulliparous female *Wistar* rats aged 5 months (Charles River laboratories, Barcelona) were randomly ovariectomized (OVX; n=13) or sham operated (SHAM; n=12). Bilateral ovariectomy was performed by standard ventral approach [24] under anesthesia with 4% sevoflurane. A sham surgery without removal of the ovaries was performed to another group of animals (SHAM). After one week of recovery, OVX and SHAM rats were separated into two sub-groups that were housed in different conditions. In one of the sub-groups, the rats were individually housed in cages with activity wheel and distance counter (floor area 800 cm^2; Tecniplast) allowing them to perform voluntary running activity (OVX+VR, n=7; SHAM+VR, n=6). In the second sub-group, animals were housed in identical conditions (OVX+C, n=6; SHAM+C, n=6) except for the running wheel in the cage. Distance traveled by each rat in the running wheel was recorded daily and body weight recorded weekly at the end of the light phase. Food intake was also recorded weekly and was determined as the difference in chow weight contained on the rack between the beginning and the end of the week. All animals were maintained in an inverted 12h light/dark cycle in a controlled environment (i.e., constant humidity and temperature). Standard rat chow and water were provided *ad libitum* throughout the experimental period.

Animal sacrifice and organs collection: Following the 9-month experimental period, all rats were anesthetized with 4% sevoflurane and sacrificed by exsanguination. Note that all animals were 14 months old at time of sacrifice. Blood was collected from *vena cava* and was later used for biochemical analysis. Parametrial, retroperitoneal, inguinal, and mesenteric fat depots were surgically dissected and weighted for quantification of intra-abdominal fat content. Both hind limbs *soleus* muscle were dissected, washed in could PBS (pH 7.2) and

weighted together. Organs weight was determined with a precision balance (resolution 0.01 mg; Kern 870). Right tibia was also collected and its length measured with a digital caliper (resolution 0.01mm, Powerfix) for correcting weight comparisons to differences in animal size. The right *soleus* muscle was removed, weighed and transversely sectioned in two samples: one processed immediately for light (LM) microscopy and the second for transmission electron microscopy (TEM). The left *soleus muscle* was minced and homogenized in a ratio of 1:10 in ice-cold extraction medium containing 50mM Tris/base and 1mM EDTA (Sigma) pH 7.4 with a Potter-Elvehjem homogenizer and pestle. The homogenate was then collected, sonicated for 1min in ice cold water (Sonorex, Bandelin Electronic) and centrifuged at 700g for 10min at 4°C. The supernatant was collected for spectrophotometric assessment of citrate synthase (CS) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity as well as type I myosin expression by Slot-Blot. Total protein content was determined by the Lowry method using BSA (Sigma) as standard.

Histology

Tissue processing for light and transmission electron microscopy: One of the right *soleus* muscle samples was fixed overnight in a solution containing 4% paraformaldehyde, 2.5% sucrose (Sigma) and 0.1% gluteraldehyde (TAAB) in PBS (pH 7.2) at 4°C, dehydrated through graded ethanol solutions, cleared in xylene and mounted in paraffin. Transverse 6µm thick sections were cut, and used for assessing fiber cross-sectional area (CSA), fibrous tissue accumulation, apoptotic myonuclei presence and type I myosin heavy chain (MyHC) expression by immunohistochemistry. The sections were analyzed with a light microscope and images recorded with a coupled digital camera (Axio Imager A1, Carl Zeiss; Germany). The remaining sample of the right *soleus* muscle was sectioned into smaller portions (≈3 mm^3) and fixed overnight in 2.5% gluteraldehyde in 0.2M sodium cacodylate buffer (Sigma; pH 7.2) at 4°C. After rinsing with 0.2M sodium cacodylate buffer they were post fixed with 2% osmium tetroxide in 0.2M sodium cacodylate, dehydrated in graded ethanol and embedded in Epon (TAAB) at 60°C until polymerization. Ultra-thin (100 nm) sections were cut in a ultramicrotome (Reichert-Jung Ultracut) with a diamond knife (ultra 45° Diatome), contrasted with uranyl acetate and lead citrate (Sigma) and analyzed with a transmission electron microscope (Zeiss EM10A) at an accelerating voltage of 60 kV. Micrographs were recorded with a coupled photographic camera.

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Soleus muscle fiber cross-sectional area (CSA): Soleus muscle sections were stained with H&E and the images analyzed with ImageJ software (NIH, Bethesda, MD). CSA was determined for each animal as the average area of 350 fibers.

Assessment of fibrous tissue accumulation: Soleus muscle sections were stained with Picrosirius red (PSR) according to the method of Sweat et al. [33] by incubation on 0.1% sirius red in saturated picric acid for 1h. Sections were then rinsed in 0.5% acetic acid, dehydrated in ethanol and cleared in xylene. PSR technique stains collagen bright red and muscle tissue yellow. Images were analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) by quantification of the percentage area covered by collagen (red) and muscle tissue (yellow).

Assessment of apoptosis in soleus muscle: The presence of apoptotic nuclei in *soleus* muscle was assayed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using a commercially available kit (*In situ* cell death detection kit AP) according to the manufacturer instructions (Roche). After deparaffinization, sections were immersed in 0.1M citrate buffer (pH 6.0) and microwave irradiated for 1 min (750W). After rinsing in could PBS, sections were first blocked with 3% BSA in 0.1M Tris-HCl (pH 7.5) for 30min at 20^{o}C and then incubated in freshly prepared TUNEL reaction solution (nucleotide mixture + terminal deoxynucleotidyl transferase) in a humidified chamber at 37^{o}C for 60 min in the dark. Negative and positive controls were simultaneously prepared by incubation with label solution only (nucleotide mixture) or by incubation with DNase I (Sigma) prior to the labeling procedure, respectively. Sections were analyzed with a fluorescent microscope coupled to a digital camera (Axio Imager A1, Carl Zeiss) and apoptotic cells were identified as brightly fluorescent in opposition to the pale green background staining.

Soleus muscle ultrastructure analysis: Morphometric analysis of *soleus* muscle ultrastructure was performed on the digitized TEM micrographs. Intermyofibrillar (IMF) and subsarcolemmal (SSM) mitochondria size (area) as well as total mitochondria density (n/μm^2) were determined using imageJ software (NIH, Bethesda, MD) considering each image magnification. Qualitative morphological analysis of sub cellular structures was also performed.

Assessment of soleus muscle type I myosin heavy chain (MyHC) expression by immunohistochemistry: After deparaffinization and rehydration, *soleus* muscle sections were rinsed in 0.1% TBS-T and non specific binding was blocked by incubation with 3% BSA in 0.1%

TBS-T for 30 min at 37°C. Sections were then incubated with mouse monoclonal anti type I myosin (M8421, Sigma) primary antibody diluted 1:100 in 0.1% TBS-T overnight in a humidified chamber. After rinsing 3x 5min in 0.1% TBS-T, sections were incubated with goat anti-mouse alkaline phosphatase conjugated secondary antibody (SC-3698, Abcam) diluted 1:100 in 0.1% TBS-T for 1h at 37°C in a humidified chamber. Detection was performed by incubation with Fast Red TR/Naphthol AS-MX Tablets (SigmaFast, Sigma). Negative controls were performed for each section by omission of the primary antibody. Counterstaining was performed with hematoxylin.

Biochemistry

Determination of serum 17β-estradiol concentration: Serum was separated from the blood samples by centrifugation at 4°C and estradiol (17β-estradiol) concentration was assayed by solid phase competitive binding ELISA using a commercially available kit (Estradiol ELISA DE2693) and a spectrophotometer (iEMS, Labsystems) according to the manufacturer recommendations (Demeditec Diagnostics) for validating the effectiveness of the OVX procedure. Briefly, 25µl of serum and 200µl of enzyme conjugate were dispensed in each plate well and incubated for 120 min at 25°C. After complete washing, 100µl of substrate solution was added to each plate well and incubated for a further 15min. Absorbance was read at 450nm after addition of 50µl stop solution. Duplicates were analyzed for each sample and triplicates for each standard. Assay range is between 9.7 to 2000 pg/mL.

MyHC I immunoblot: Semi-quantification of MyHC I content was performed by Slot Blot as described previously [14]. Briefly, samples of *soleus* muscle homogenate containing 10µg of protein were loaded into a nitrocellulose membrane (Hybond; Amersham Biosciences) with a slot blot filtration manifold device (Hybri-Slot; Gibco BRL) coupled to a vacuum pump (KNF Neuberger). The membrane was then blocked in 5% non-fat dried milk in 0.1% TBS-T for 1h, incubated with primary antibody (Mouse monoclonal anti type I myosin; M8421, Sigma) diluted 1:1000 in 5% non-fat dried milk in 0.1% TBS-T for 2h, washed 3x 5 min in TBS-T and then incubated with secondary antibody (Rabbit polyclonal to Mouse IgG HRP; ab6728, Abcam) conjugated with horseradish peroxidase diluted 1:1000 in 5% non-fat dried milk in 0.1% TBS-T for 2h. The blots were developed by enhanced chemiluminescence according to the manufacturer instructions (Amersham Pharmacia Biotech), followed by exposure to a photographic film (Kodac Biomax Ligth Film, Sigma). The films were then analyzed with ImageJ software (NIH, Bethesda, MD) using the “Integrated Density” option to measure the intensity

of the auto radiographic signal inside a boundary drawn around the bands detected in the films after background subtraction.

Determination of citrate synthase (CS) activity: CS activity was determined as described previously [7] by the spectrophotometric measurement at 412nm of the amount of 5,5-dithio-bis(2-nitrobenzoate) that reacted with acetyl-coenzyme A (CoA) after its release from the reaction of acetyl-CoA with oxaloacetate. Brifly 150µl of ultrapure water, 20µl of freshly prepared assay buffer containing 1.0mM DTNB and 1% Triton X-100 (pH 8.1), and 20µl of the *soleus* homogenate were dispensed to each plate well. The absorbance was read for 5min with 15 seconds interval in a spectrophotometer (iEMS, Labsystems) at 37^{o}C following the addition of 10µl of 10mM oxaloacetate. The activity was calculated using CS from porcine heart (C3260, Sigma) as standard and expressed per mg of protein content.

Determination of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity: GAPDH activity was measured as described previously [36] as an increase in absorption at 340nm following the reduction of NAD. Briefly freshly prepared 173µl of assay buffer containing 15mM sodium pyrophosphate and 30mM sodium arsenate pH 8.5, 6.6µl of 7.5 mM NAD, 6.6µl of 100mM DTT (Sigma) and 6.6µl of the *soleus* homogenate were dispensed to each plate well. Absorbance was read at 340 nm for 5 min with 15 seconds interval in a spectrophotometer (iEMS, Labsystems) at 37^{o}C following the addition of 6.6µl of 7.5mM glyceraldehyde 3-phosphate solution. The activity was calculated using GAPDH from rabbit muscle (C2267, Sigma) as standard and expressed per mg of protein content.

Statistical analysis: The Kolmogorov-Smirnov test was used to investigate within-group normality for a given variable. Levene´s test was used to assess homogeneity of variance. Comparisons between groups were performed by two-way analysis of variance (ANOVA) with Bonferroni *post hoc* test if normality and equality of variance existed. When normality or equality of variance were not verified, root square transformation was employed (intra-abdominal fat weight) to reestablish the necessary assumptions for ANOVA. Comparisons between groups regarding mitochondria size and number were performed with the Mann-Whithney non-parametric test due to distribution lack of normality. Differences were considered significant at $p<0.05$ and results are presented as mean ± standard deviation (SD). Data was analyzed with SPSS 17.0. Post hoc analysis of the statistical power achieved for the number of animals used was performed with G*Power 3.1 software and was found to be between 0.95 and 0.98 for all variables assayed.

Results

Serum 17β-estradiol: Mean serum 17β-estradiol concentration in OVX+C (20.4±5.69 pg/mL) and OVX+VR (16.7±4.78 pg/mL) groups was significantly lower compared to SHAM+VR (86.7±16.57 pg/mL) and SHAM+C (82.4 ± 19.51 pg/mL) groups. No significant differences existed between OVX+C and OVX+VR and between SHAM+VR and SHAM+C.

Physical activity: During the first week of the experiments the average daily distance traveled by OVX+VR and SHAM+VR animals were similar (1.02±0.383Km.day^{-1} *vs*. 1.38±0.631Km.day^{-1} respectively, p=0.294; Figure 1A). However, beginning in week two, the difference in physical activity began to differ markedly between the experimental groups. Indeed, by the end of the 9 months of differential housing SHAM+VR animals accumulated a 10-fold higher running distance compared to OVX+VR animals (1449.7±308.98Km *vs*. 146.0±31.46Km respectively; Figure 1B).

Body weight variation and food intake: Detailed information is presented in table 1.

At the beginning of these experiments, body weight did not differ between the experimental groups. However, over time all animals increased body weight significantly (p≤0.003) with larger body weight increases in OVX animals compared to their SHAM counterparts. Further, body weight increases in the OVX+C were greater than the OVX+VR animals (p<0.05). Regardless of their higher body weights, food intake was similar between SHAM and OVX animals but significantly higher (p<0.01) in exercised than in sedentary animals. At the time of sacrifice, while OVX+VR body weight was significantly higher than that of SHAM+VR (400.9±36.61g *vs.* 303.2±41.27g, p<0.01) no differences were identified between OVX+C and SHAM+C groups (363.5±31.79g *vs.* 324.7±39.67g, p=0.087). Intra-abdominal fat mass was also higher in OVX+VR animals compared to SHAM+VR (33.6±10.15g *vs.* 10.5±9.27g, p<0.01) but not between OVX+C and SHAM+C animals (30.9±7.77g *vs.* 25.3±7.59g, p=0.285).

Soleus muscle weight was significantly higher in exercised animals compared to sedentary controls (p<0.05) but no differences existed between OVX+VR and SHAM+VR (p=0.5) as well as between OVX+C and SHAM+C (p=1.0).

Soleus muscle fiber CSA and protein content: Average *soleus muscle* fiber CSA was 1913.2±269.10µm^2, 1048.5±315.81µm^2, 2920.4±805.39µm^2 and 1175.6±395.59µm^2 for OVX+VR, OVX+C, SHAM+VR and SHAM+C groups, respectively. A significantly higher *soleus* muscle fiber CSA was identified in SHAM+VR animals compared to OVX+VR animals but there were no significant differences between OVX+C and SHAM+C (Figure 2). Fiber CSA was

significantly correlated with the amount of running each animal performed on the wheel ($r=0.765$; $p<0.05$). To investigate if fiber size differences reflected true anabolic gains or just increases in non-protein content (i.e. edema), total protein content of the left soleus muscle was determined and expressed as percentage of muscle wet weight. Total protein content was 15.3±3.08%, 17.4±2.85%, 15.0±2.24% and 17.1±2.40% for OVX+VR, OVX+C, SHAM+VR and SHAM+C groups, respectively. No significant differences in total protein existed between these experimental groups ($p\geq0.757$).

Myofiber apoptosis and fibrous tissue expression: No signs of apoptotic nuclei were identified in *soleus* muscle fibers of any experimental group despite the presence of other positively stained interstitial cells (Figure 3A). Muscle collagen content was greater in both the OVX and SHAM control groups compared to their exercised counterparts (Figure 3B, C). No differences in muscle collagen content existed between animals housed in the same conditions.

Soleus muscle ultrastructure: No significant differences existed in soleus muscle fiber mitochondria density (Figure 4) between the experimental groups despite the slightly higher mean values in exercised animals (Mean±SD): OVX+VR =0.30±0.133, μm^2, SHAM+VR =0.28±0.094, μm^2, OVX =0.21±0.071, and SHAM =0.20±0.029, μm^2. Further, no differences existed in IMF mitochondrial size between the groups (Figure 4J). In contrast, SSM mitochondria were significantly larger in the soleus muscle of SHAM+VR compared to OVX+VR (Figure 4B, F). No differences in SSM mitochondria size existed between the two sedentary groups (Figure 4D, H) although SSM mitochondria were significantly smaller in the sedentary groups compared to both exercise groups (Figure 4K). Independent of their estrogen status, exercised animals also showed abundant glycogen granules, signs of satellite cell activation (Figure 5A), increased myonuclei transcriptional activity (Figure 5C) and myofibril splitting (Figure 4E). Several OVX+VR and SHAM+VR animals also displayed frequent secondary lysosomes (Figure 5B, D), often near large aggregates of SSM mitochondria. In contrast, these features were in general not observed in both control groups. In turn, muscles from sedentary control animals showed increased levels of both adipose and fibrous tissue (Figure 4G) as well as thinner myofibrils with evidence of sarcomeric disorganization (Figure 4C).

Enzymatic profile: Soleus muscle CS activity was 3.99±0.784, 4.99±0.706, 4.32±1.514 and 5.18±0.828 μmol/min/mg in OVX+C, OVX+VR, SHAM+C and SHAM+VR groups respectively. Although soleus muscle CS activity tended to be higher in the exercise animals compared to

sedentary, these differences did not reach significance (p≥0.052). GAPDH activity in the soleus muscle was 535±124.1, 521±107.1, 531±110.5 and 569±187.6 nmol/min/mg in OVX+C, OVX+VR, SHAM+C and SHAM+VR groups, respectively. No significant differences existed between the experimental groups.

Soleus muscle MyHC I expression: The immunoblot analysis (Figure 6A) showed that *soleus* muscle MyHC I was over expressed in OVX compared to SHAM animals independently of their housing conditions. Access to the running wheel significantly influenced MyHC I expression in SHAM but not in OVX animals. Further, no differences in MyHC I expression existed between OVX+VR and OVX+C animals. SHAM animals MyHC I was over expressed in those with access to the running wheel. *In situ* findings of MyHC I expression assayed by immunohistochemistry (Figure 6B) also revealed a higher number of muscle fibers positively stained in OVX animals compared to SHAM. Note, however, that a quantitative analysis of muscle fibers expressing MyHC I isoforms was not performed since the majority of the fibers were found to be hybrid, revealing several degrees of affinity with the antibody against type I myosin.

Discussion

Overview of major findings

These experiments provide three important findings. First, ovariectomy significantly reduces voluntary physical activity in rats. Second, our data also reveal that ovariectomy-induced sarcopenia in female rats is not directly linked to the loss of ovarian hormones (OH) but appears to be due, at least in part, to a decrease in voluntary physical activity. Finally our results indicate that the surgical removal of the ovaries significantly changes the myosin isoform profile within the soleus muscle as the expression of MyHC I was significantly increased in both exercised and control OVX animals compared to SHAM counterparts. A brief discussion of each of these major findings follows.

Ovariectomy reduces voluntary physical activity in rats

Our findings clearly demonstrated that ovariectomy greatly decreases voluntary physical activity in rats. Indeed, compared to the OVX+VR animals, the sham operated animals (SHAM+VR) ran, on average, a 10-fold greater distance each day during the 9-month experimental period. For example, the OVX+VR animals averaged only ≈560 meters/day whereas the SHAM+VR animals traveled an average of ≈ 5580 meters/day during the duration of the study.

Note that previous studies have also shown that motor activity is influenced by circulating estrogen levels [1,8,11,16,31]. Nonetheless, most studies have described variations in physical activity during relatively short experimental periods (i.e., days to 12-weeks) following estrogen loss [23] [22]. Therefore, the current study is unique because we investigated animal activity patterns following ovariectomy for long time periods (i.e., 36 weeks). Moreover, by investigating the long-term effects of ovariectomy, our results reveal that the level of inactivity associated with the loss of ovarian hormones is greater than previous reports that investigated activity patterns during relatively short time periods following ovariectomy [11,16]. Finally, our results reveal that the reduced voluntary physical activity associated with ovariectomy lasted throughout the entire duration of our study, suggesting that the effects induced by OH loss are permanent.

Ovariectomy does not alter muscle fiber size

To determine if the loss of ovarian hormones has a direct effect on muscle structural and biochemical properties or if these changes are a consequence of muscle disuse due to decreases in voluntary motor activity, we analyzed muscle structure and metabolic profile in

animals with and without access to running wheels. We reasoned that muscle differences between sham operated and ovariectomized animals exposed to standard housing would reflect alterations in skeletal muscle structure resulting from OH loss while differences between animals housed with running wheels would reveal additional muscle alterations that occur due to differences in voluntary physical activity levels. We specifically selected the soleus muscle for study because this muscle has been widely used by others for the study of OH loss effects on skeletal muscle [16,20,21]; hence, this enables a comparison of the current data with findings from previous studies.

Our results reveal that the soleus muscle weight and fiber CSA did not differ between the OVX and SHAM control animals (Table 1; Figure 2). These results indicate that the absence of OH did not impact soleus muscle weight and suggests that estrogen is not a requirement to maintain muscle mass. Further, our data indicate that when given access to the running wheel, soleus muscle mass of OVX animals increased significantly and was not different from that of SHAM+VR animals, indicating that exercise was effective in inducing an anabolic response. This finding further supports the concept that loss of OH *per se* does not lead to skeletal muscle atrophy, since without the anabolic stimulus provided by exercise, CSA was identical between OVX+C and SHAM+C animals. Nonetheless, soleus muscle fibers CSA from OVX+VR animals were significantly smaller compared to the CSA of soleus muscle fibers from the SHAM+VR animals. Interestingly, the soleus muscle fiber CSA of these two groups of animals were highly correlated ($r=0.765$; $p<0.05$) with the daily distance traveled in the running wheel. Again, these results are consistent with the concept that OH per se, does not directly regulate muscle fiber size but rather muscular activity plays a significant role in determining muscle mass during aging.

It is noteworthy that a previous study has reported that ovariectomy results in an increase skeletal muscle water content [18]. Therefore, we analyzed the total protein content of the soleus muscle and determined that the ratio of protein content/muscle wet weight was similar between all groups. These findings indicate that our group differences in muscle weights and fiber CSA's were not due to differences in muscle water content.

Further, since variations in estrogen status could also influence food intake [10] which could impact skeletal muscle mass independently of receptor mediated estrogen effects, we recorded the animals food intake throughout the experimental period. Our results show that OVX and SHAM animals ingested the same amount of food when housed in equivalent conditions. Therefore, differences in food intake did not contribute to the variance in muscle mass between the experimental groups. In addition, when access to the running wheel was

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provided, both OVX+VR and SHAM+VR animals increased their food intake by an identical ≈28% compared to control animals.

Ovariectomy does not alter muscle structure

Previous studies provide evidence that estrogen exerts anti-apoptotic effects in skeletal muscle, which raises the possibility that OVX could impact soleus muscle structure and size by increasing the rate of apoptotic cell death [4,35]. We therefore analyzed soleus muscle fibers for the presence of myonuclei undergoing apoptosis using the TUNEL staining method. Our results revealed no signs of myonuclei undergoing cell death in any of the experimental groups indicating that OH absence was not associated with increases in apoptotic nuclei at the time of the animals sacrifice (Figure 3).

Further, a previous report has demonstrated that estrogen significantly affects the compositions of the extracellular matrix in cardiac myocytes by inhibiting fibrous tissue accumulation [38]. Others have also reported that estrogen deficiency leads to an increase in the collagen/muscle ratio in the rat urinary tract [27] and that estrogen replacement up regulates the synthesis of MMP-2 in mice mesangial cells [12]. Hence, to determine if loss of ovarian hormone affected the extracellular matrix composition we quantified the fibrous tissue accumulation in the soleus muscle by staining with picrosirius red. Our results indicate that OH absence was not associated with an increase in fibrous tissue accumulation as no differences existed between OVX and SHAM animals housed in the same conditions (Figure 3). Sedentary behavior, but not ovariectomy, influenced fibrous tissue accumulation with both OVX and SHAM control animals displaying increases in collagen amount when compared to VR counterparts (Figure 3). Similar results have been reported by others in experiments investigating the impact of inactivity on muscle tissue connective tissue [15].

Our examination of the ultrastructure of muscle also indicates that loss of OH did not impact the structure of the soleus muscle (Figures 4, 5). However, both the OVX and SHAM animals with access to the running wheel showed signs of metabolic and structural adaptations induced by regular physical activity, which included increased SSM mitochondria size, increased presence of glycogen granules and signs of satellite cell activation as well as signs of highly active protein synthesis, as evidenced by the presence myonuclei with multiple nucleoli and prominent endoplasmic reticulum. Consistent with these ultrastructural results, our findings in soleus muscle sections stained with H&E (Figure 2) revealed myofibers with central nuclei suggesting an increase in muscle regeneration. Note, however, that central nuclei were not observed in muscle fibers of either OVX or SHAM control animals. This indicates that exercise was the main stimulus for the increased anabolic activity observed in

muscle cells, and that in the absence of exercise, anabolic activity was similar between experimental groups independently of OH status.

Finally, previous studies have reported that mitochondria are an estrogen sensitive organelle and that estrogen administration to OVX animals enhances respiratory chain activity and antioxidant enzyme expression [25]. Therefore, we quantitatively evaluated mitochondria size and density to determine if a lack of OH alters mitochondrial size in skeletal muscle. Our analysis revealed that the SSM mitochondria size was reduced in both control groups, independent of the OH status. Therefore, mitochondria size and density was unaffected by OVX. Moreover, lack of OH did not influence SSM size in OVX animals with access to the running wheel.

Ovariectomy increases the abundance of myosin heavy chain type I in muscle

Our results reveal an increase in MyHC I expression occurred in the soleus muscle of both control and running OVX animals in comparison to their SHAM counterparts (Figure 6). These results indicate that a loss of OH stimulated a shift in soleus muscle contractile protein toward an increase in MyHC I abundance in the muscle fibers. Previous studies on cardiac muscle also show that a shift in MyHC isoform expression from fast to slow occurs following ovariectomy [28] and that this shift is reversed with estrogen replacement therapy [29]. Nonetheless, studies investigating the impact OH on skeletal muscle have been more inconsistent with some reports describing a fast-to-slow shift in muscle phenotype whereas others report no changes in myosin heavy chain isoform [16,19]. The reasons for this variation in the literature remain unclear.

Interestingly, despite the increased expression of MyHC I isoform identified in OVX animals, this was not accompanied by notorious metabolic profile changes as differences in soleus muscle CS and GAPDH activity did not exist between OVX and SHAM in both the sedentary and running animals. We interpret these findings as an indication that the lack of OH does not have a major impact on the expression of bioenergetic enzymes in skeletal muscle.

Summary and Conclusions

These experiments provide several new and significant findings. Foremost, ovariectomy results in a large and permanent decrease in voluntary physical activity in rodents. Further, our results indicate that ovariectomy-induced sarcopenia in female rats is not due to the loss of ovarian hormones (OH) per se, but appears to be due, at least in part, to a decrease in voluntary physical activity. Furthermore, our experiments also reveal that the surgical removal of the ovaries significantly changes the myosin isoform profile in skeletal muscle as the

abundance of MyHC I was significantly increased in muscle of both exercised and control OVX animals compared to SHAM counterparts.

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List of Figure Captions

Figure 1. Voluntary motor activity recorded on the running wheel. (A) Running well activity of OVX+VR and SHAM+VR rats was continuously monitored throughout the 9 months of the experimental procedure. As soon as after the first week following surgery, distance traveled by OVX+VR animals was found to be always significantly lower than SHAM counterparts (*p<0.05). (B) Total running distance was 10-fold higher in SAM+VR than in OVX+VR animals. C-control; VR-voluntary running; OVX-ovariectomized; SHAM-sham operated. *Significant differences between groups (p<0.05). Data are mean ± standard deviation.

Figure 2. Average *soleus* muscle myofiber cross sectional area (CSA). (A) Micrographs of *soleus* muscle fibers sectioned transversely and stained with H&E. A higher fiber CSA is evident in both OVX and SHAM running animals. Arrows point out myofibers whith central nucleus. Bars are 50µm. (B) Quantitative assessment of soleus muscle fiber CSA. C-control; VR-voluntary running; OVX-ovariectomized; SHAM-sham operated. *Significant differences between groups (p<0.05).

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Figure 4. Soleus muscle ultrastructure assayed by transmission electron microscopy (TEM). It was possible to identify, in running animals that were either OVX (B) or SHAM operated (F), large aggregates of subsarcolemmal (SSM) mitochondria, clearly contrasting with SSM mitochondria seen in OVX (D) or SHAM sedentary controls (H) which were smaller and sparser. Quantitative analysis of mitochondria number per square micrometer (I), intermyofibrillar (IMF) mitochondria (J) and SSM mitochondria median size (K). (E) Arrows highlight signs of myofibril splitting in SHAM+VR animals. (G) Arrows highlight areas of increased fibrous tissue accumulation. (C) Arrows highlight sarcomeres with disorganized

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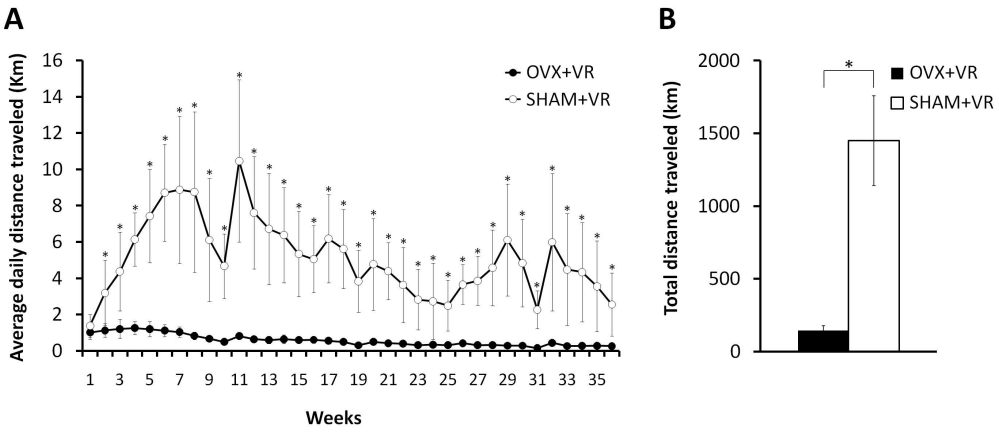
Figure 5. *Soleus* muscle TEM photomicrographs from OVX+VR (upper panel) and SHAM+VR (lower panel) animals. (A) The presence of a prominent nucleolus and rough endoplasmic reticulum (arrow head), as well as an increased cytoplasm/nucleus size ratio indicates satellite cell activation. Arrows point out places were the satellite cell plasma membrane has already disappeared demonstrating that fusion with the underling myofiber is occurring. (C) Myofiber nucleus with multiple nucleoli suggesting highly active translational activity. Secondary lysosomes were frequently distinguishable in both OVX and SHAM running animals. B and D micrographs show the presence of secondary lysosomes between subsarcolemmal mitochondria which in turn show no signs of compromised structure.

Figure 6. S*oleus* muscle myosin heavy chain I (MyHC I) isoform expression. (A) Slot blots with graphic representation of MyHC I protein expression. (B) MyHC I expression assayed by immunohistochemistry. In the upper micrograph it is possible to identify muscle fibers with high affinity for MyHC I antibody (arrohead) together with fibers showing no affinity what so ever (black arrows) who were therefore assumed to be composed mainly of other MyHC isoforms. Most fibers however displayed some intermediate degree of affinity with the antibody (white arrows) and were considered to be hybrid regarding MyHC isoform expression. The lower photomicrograph shows a negative control s*oleus* muscle section with some artefactual staining due to alkaline phosphatase activity on blood vessels. C-control; VR-voluntary running; OVX-ovariectomized; SHAM-sham operated; AU–arbitrary units. *Significant differences between groups ($p<0.05$). Bars correspond to 50µm.



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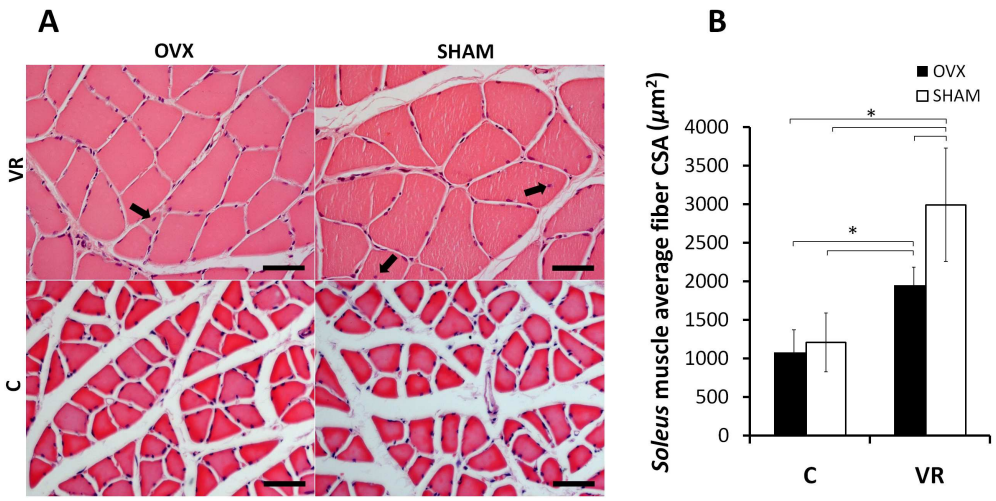

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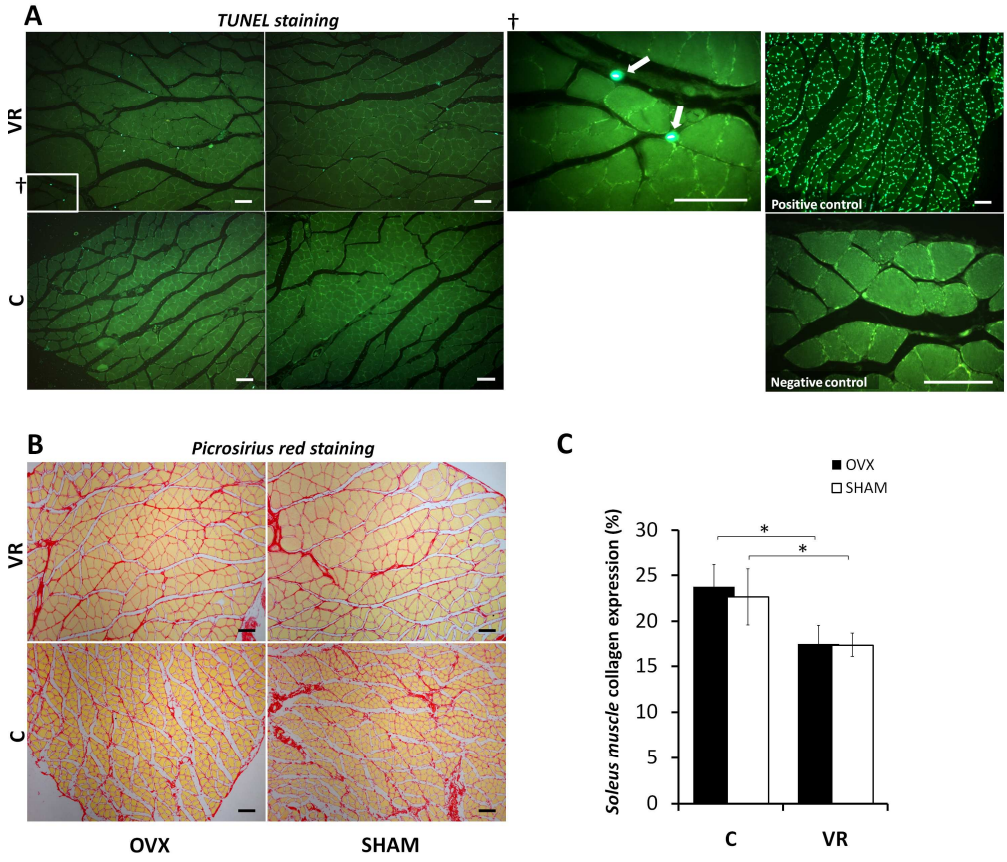

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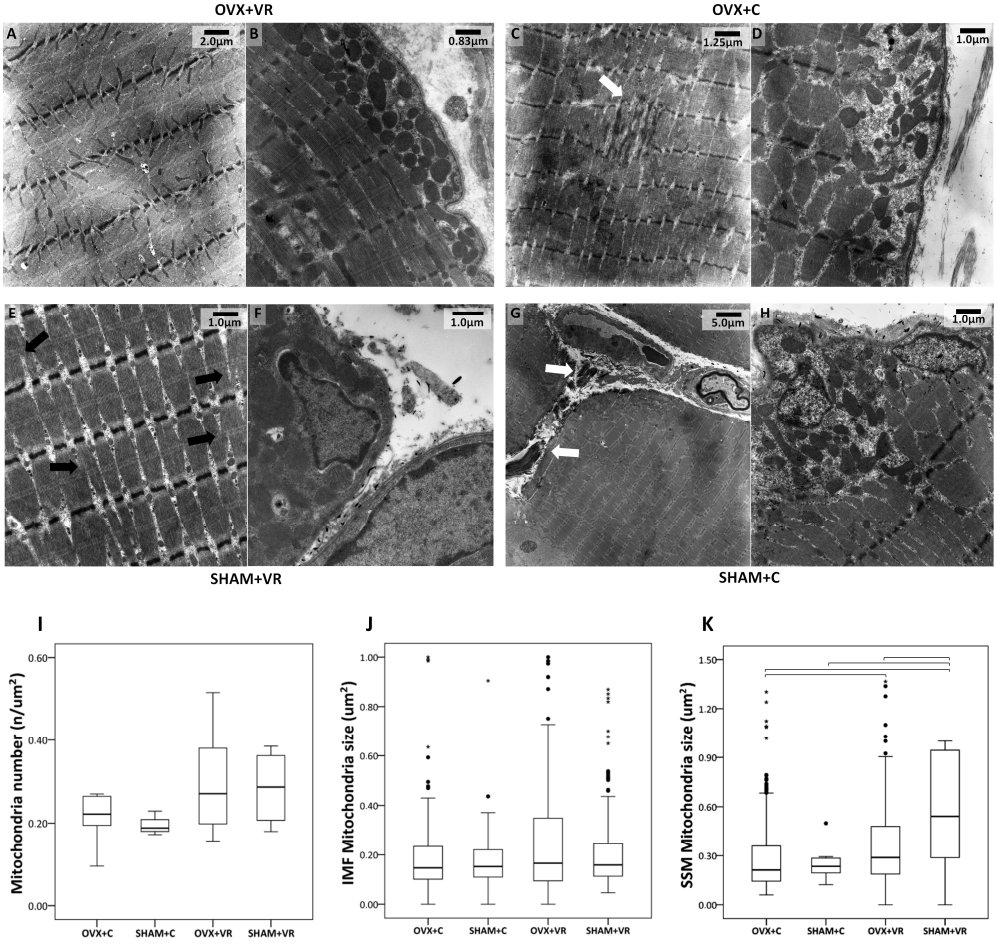

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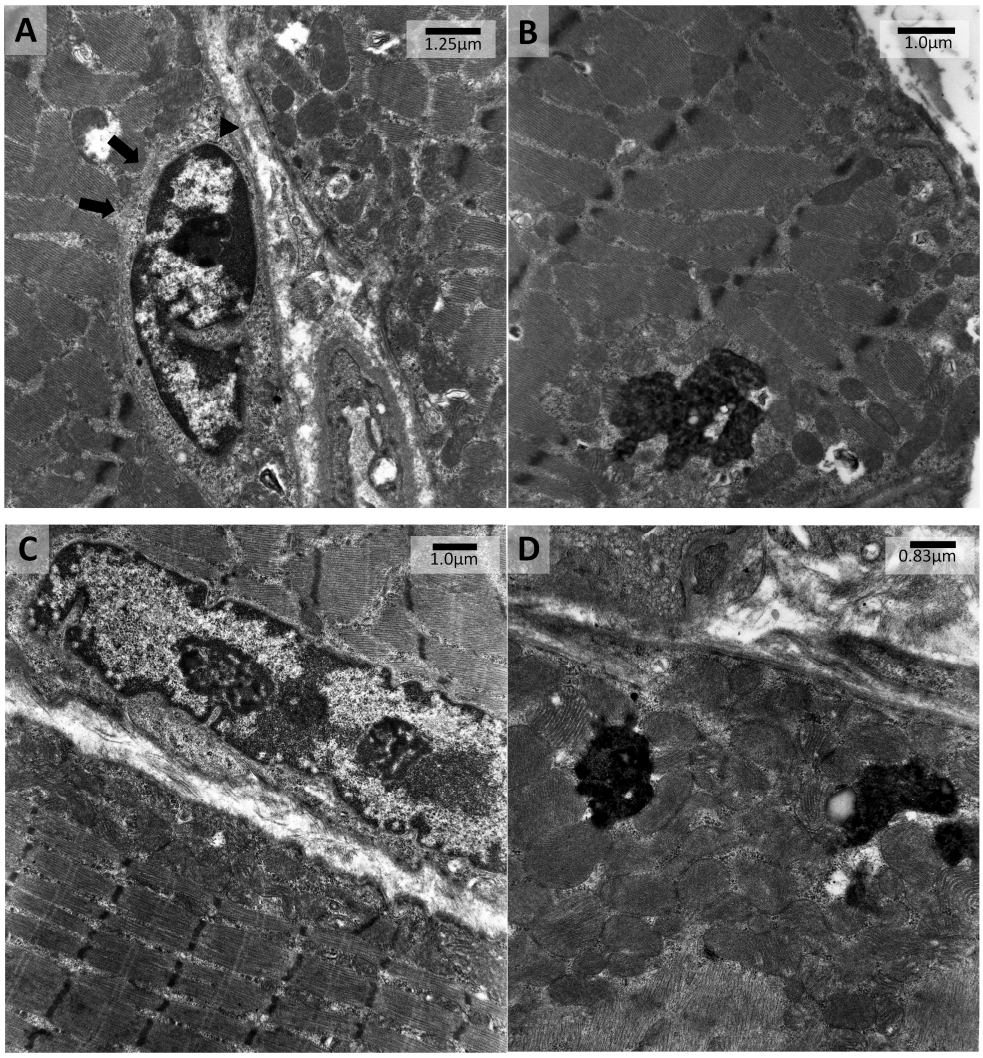

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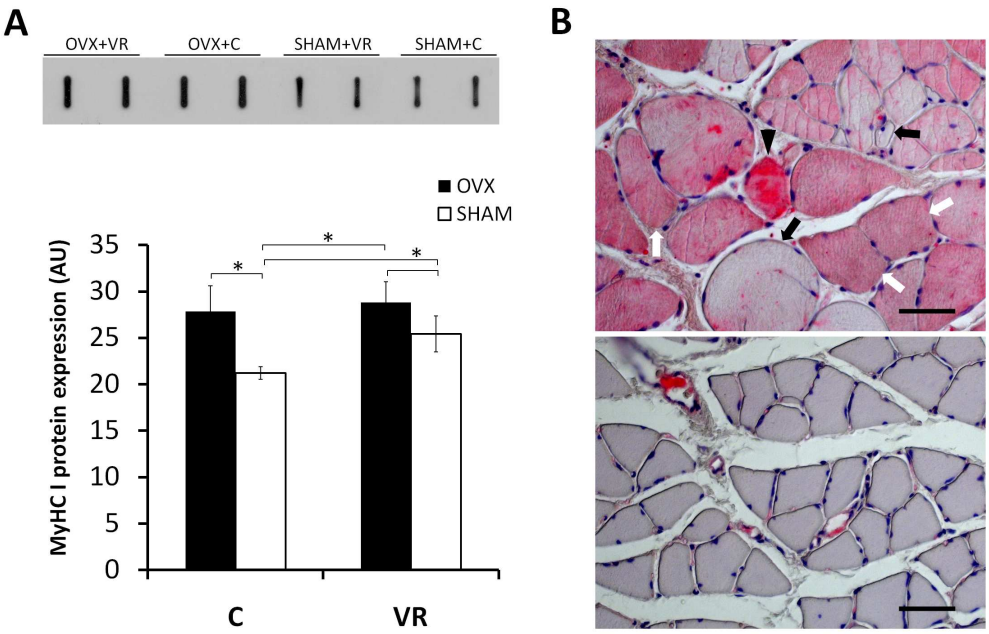

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Table 1. Food intake, whole body, intra-abdominal fat and *soleus* muscle weights.

Weight	OVX+C	OVX+VR	SHAM+C	SHAM+VR
Whole Body				
Initial (g)	267.1 ± 28.72	260.3 ± 18.32	269.8 ± 16.70	241.8 ± 9.61
At sacrifice (g)	363.5 ± 31.79	400.9 ± 36.61 †	324.7 ± 39.67	303.2 ± 41.27
Variation (%)	35.4 ± 7.13 †	54.1 ± 11.27 *†	21.0 ± 11.19	25.3 ± 15.85
Intra-abdominal fat (g)	30.9 ± 7.77	33.6 ± .10.15 †	25.3 ± 7.59	10.5 ± 9.27 *
Total food intake (g)	4,225 ± 230.8	5,169 ± 306.0 *	4,311 ± 439.2	5,408 ± 599.2 *
Weekly food intake (g)	115 ± 5.5	148 ± 8.7 *	118 ± 11.4	151 ± 16.3 *
Soleus muscle (mg)	210 ± 20.6	261 ± 31.3 *	211 ± 23.5	288 ± 26.6 *

* p<0.05 versus SED counterpart.

† p<0.05 versus SHAM counterpart.

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Study 2

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Lifelong Sedentary Behaviour and Femur Structure

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Key words
- senile osteoporosis
- histomorphometry
- ageing
- lifelong physical activity
- osteocytes

Abstract

The aim of the present study was to analyze the lifelong differences of femur structure in sedentary and physically active animal models. Thirty male C57BL/6 mice, 2 months old, were either: i) housed in cages with running wheel (AA; n = 10), ii) housed in cages without running wheel (AS; n = 10), iii) or sacrificed without intervention (Y; n = 10). AA and AS animals were sacrificed after 23 months of housing. Right femur structure was analyzed in all animals by histomorphometry. Significant differences in several microarchitectural parameters of cancellous and cortical bone were identified between Y mice and both groups of aged mice, as well as between AA and AS groups. Lifelong physically active mice had significantly higher cancellous bone surface (Cn.BS) and trabecular number (Tb.N) and decreased trabecular separation (Tb.Sp) at both epiphyses when compared to AS animals. No differences were observed between Y and AA groups regarding osteocyte number (N.Ot) despite its significant reduction in AS animals, suggesting that age alone was not a cause for decreases in N.Ot. Our results suggest that the reduced bone quality observed in the elderly is not only a consequence of age but also of lack of physical activity since sedentary behaviour significantly aggravated the degenerative age-related bone differences.

Introduction

The overall improvements in health care services have allowed life expectancy to be enhanced in many developed countries. Due to the increase in the elder population proportion [25] it is expected that the occurrence of degenerative diseases will rise, making them a major cause of concern in these countries [7].

Osteoporotic bone fractures are among the most distressing health concerns in the elderly [29] being both a clinical and public health problem, as they are associated with increased mortality [24], morbidity [21] and health care expenditures [20]. Although several risk factors may contribute to bone fragility [28], epidemiological studies show that advanced age is the major common risk factor to comprise a bone fracture [1, 18, 35].

Given the magnitude of the osteoporosis problem, the most cost-effective strategy should be prevention of fracture occurrence. Exercise is shown to be effective in preventing fracture occurrence, being for that reason included in several health guidelines for fracture prevention in individuals who have already osteoporosis [14], or as a way to prevent osteoporosis onset in the young by increasing peak bone mineral density [13]. The evidence that exercise is an anabolic stimulus for bone formation comes from both human and animal studies, where parameters suggesting increased resistance to fracture [34] are found in bones stimulated by mechanical strain [37] and tend to be absent in those that lack stimulation, namely following microgravity [17] or prolonged immobilization [4]. Exercise interventions have also shown to reverse the deleterious effects of reduced mechanical strain in bone [16].

Despite being poorly understood, the progressive decline in physical activity with age is a well-described biological phenomenon [15]. Considering the role of mechanical strain for bone health, it is therefore unknown whether the progressive age-related loss of skeletal quality results from the ageing process *per se* or if it reflects the age-related decrease in physical activity levels. Studies performed up to now have mainly focused on the effects of an exercise program during a determined period of time. Although these stud-

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ies have demonstrated the importance of mechanical stimulation for bone health, little is known about the effects that lifelong sedentary behaviour has on bone health and the extent to which skeletal fragility could be a consequence of disuse. To address this problem, it would be necessary to determine bone quality differences between subjects that have an active pattern of physical activity and subjects that have a sedentary lifestyle.

The majority of the studies performed concerning this subject, both in humans as well as in animal models, employ intermittent exercise interventions. Subjects that participate in clinical trials for assessing the influence of an exercise intervention are submitted to a program of physical activity, the remaining daily activities being roughly monitored. In these cases, the time in exercise corresponds to just a minor fraction of the daily behaviour, impairing the possibility to understand the real scope that a natural physical activity pattern would have on bone health. Additionally, the outcomes of the exercise intervention are matched to control individuals, in which habitual physical activity is difficult to control and therefore frequently suffer from bias contamination. Similarly, studies using animal models, although being able to more accurately control physical activity, generally have similar problems. Animals are housed in standard cages from which they are removed whenever the exercise protocol is to be performed. This leads to a situation where the animal is unable to have a wild-like activity pattern since it is constrained to the cage dimensions, having therefore a sedentary behaviour for most of the time. Moreover, the use of forced-exercise protocols is usual. These have been shown to increase stress levels with their disadvantageous related outcomes [22], namely the increase in glucocorticoid production [9].

We propose an alternative approach to investigate if age is the sole cause for the skeletal deterioration in the elderly, or if reduced physical activity is also a major determinant. To accomplish this, a group of experimental animals was housed for lifelong in conventional cages, therefore being constrained to a sedentary behaviour. Histomorphometric properties observed in these animals were compared to young animals and to a group of animals that were also housed for life, but in cages equipped with running wheels, allowing them to be physically active, mimicking the wild-like voluntary physical activity pattern. Therefore it might be assumed that the skeletal features identified in animals that were allowed to freely exercise resulted from ageing *per se* while the skeletal features of animals housed in sedentary conditions resulted from both the ageing process and lack of physical activity. As a result, comparison between these 2 groups allowed us to identify the effects of lifelong sedentary behaviour on bone tissue. In order to support our experimental model and take into account the muscle-bone interaction [6], we also assessed the effect of the two different housing conditions on skeletal muscle properties.

Materials and Methods

Animal handling and experimental design

Thirty male C57BL/6 mice, 2 months of age, were purchased from Charles River Laboratories (Barcelona) and randomly assigned into 3 experimental groups: young (Y; n = 10), aged sedentary (AS; n = 10) and aged active (AA; n = 10). After one week of quarantine following arrival, all animals from the Y group were sacrificed by a lethal intraperitoneal injection of ketamine and xylazine. The remaining animals were housed individually in standard mice cages (size: L35 × W20 × H13 cm; Tecniplast, Italy). The cages of the AA mice were additionally equipped with a running wheel (diameter 23 cm; lane width 5 cm; Tecniplast) and revolution counter, allowing them to freely exercise while the travelled distance was monitored. Voluntary wheel running was used previously to study exercise induced bone adaptations [23] and effects of lifelong exercise on the attenuation of age related conditions [5]. Both AS and AA animals were sacrificed as described above at 25 months of age. During the 23 months of the experimental period, animals of both groups were maintained at constant environmental temperature (20 °C) on a 12 h inverted light cycle and fed with standard rodent chow (A04 chow diet, Scientific Animal Food & Engineering) and water *ad libitum*. Body weights were recorded at the beginning of the experimental period and further in each trimester until sacrifice. Male C57BL/6 mice were selected as models in this study because their age-related changes in skeletal mass and architecture are similar to those seen in human ageing [11]. All animal interventions were approved by the local ethics committee and were in accordance with the ethical standards of the IJSM [12].

Tissue processing for light microscopy

Immediately after sacrifice, the animals were weighed on a precision scale (Kern 440-35N) and the right femur excised, cleared of surrounding soft tissue and fixed in a solution containing 4% paraformaldehyde (Sigma; St Louis), 2.5%. sucrose (Sigma; St Louis) and 0.1% gluteraldehyde (TAAB; UK) in PBS (pH 7.2) at 4 °C. Following fixation, the femur samples were decalcified in 10% EDTA (Sigma; St Louis) in PBS (pH 7.2) at 4 °C. The decalcification solution was changed daily and calcium presence was assayed by precipitate formation after addition of 5% ammonium oxalate monohydrate solution (Sigma; St Louis). Absence of precipitate formation indicated complete decalcification.

After decalcification, the femurs were sectioned into proximal epiphysis, diaphysis and distal epiphysis with aid of a graticule and a stereomicroscope (SZ30, Olympus). Each anatomical region was further dehydrated through graded ethanol solutions and embedded in paraffin blocks. Seven to 9 sections from each anatomical region (6 µm thick), distanced 30 µm apart were cut on a Leica 2125 rotary microtome (Leica Microsystems Inc). Sectioning of the diaphysis was performed in the transverse plane, at half length of the bone, whilst both epiphyses were sectioned in the frontal plane being the mid region indicated by the fovea in the proximal epiphysis and by the greater intercondylar width in the distal epiphysis. Sections were stained with hematoxylin and eosin.

Bone histomorphometry

Sections were viewed under a light microscope (Axio Imager A1, Carl Zeiss; Germany) with a 2.5× (both epiphysis) or 5× (diaphysis) magnification objective and images captured by a coupled digital camera with Axio Vision 4.7 software. Obtained images were then analyzed with ImageJ software to quantify standard geometrical and microarchitectural parameters in each region. In both epiphyses histomorphometric analysis was performed within a 3 mm length region between both endocortical surfaces starting 100 µm below the growth plate line. Histomorphometric parameters analyzed in both epiphysis included: cortical thickness (Ct.Th; µm), tissue volume (TV; mm^3), absolute cancellous bone volume (Cn.BV; mm^3), absolute cancellous bone surface (Cn.BS; mm^2), relative cancellous bone volume (Cn.BV/

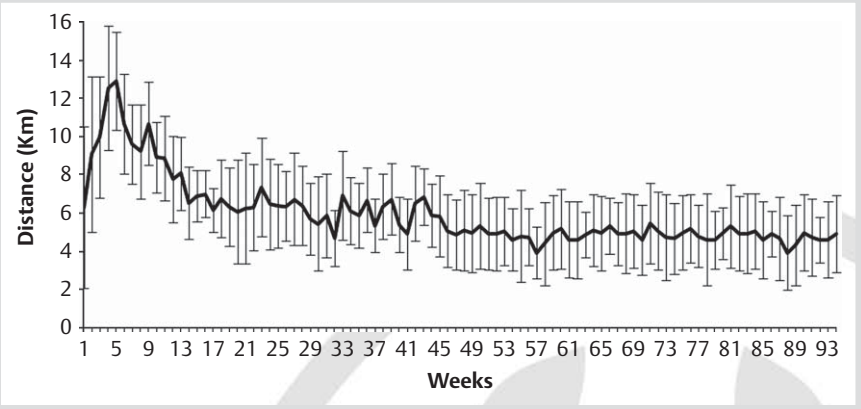

Fig. 1 Voluntary running activity of mice from the aged active (AA) group was continuously monitored in running-wheel activity cages for 23 months. Data represents the average distance (Km) that animals travelled per day in each week during the 23 months period.

TV; %), relative cancellous bone surface (Cn.BS/TV; mm^2/mm^3), trabecular thickness (Tb.Th = 2/(BS/BV); µm), trabecular number (Tb.N = (BV/TV)/Tb.Th;/mm) and trabecular separation (Tb.Sp = (1/Tb.N)-Tb.Th); mm). Histomorphometric parameters analyzed in each mid-diaphysis cross-section included: tissue volume (TV; mm^3), cortical bone volume (Ct.BV; mm^3), periosteal bone surface (Ps.BS; mm^2), marrow volume (Ma.V; mm^3), marrow perimeter (Ma.Pm; mm), bone diameter (B.Dm; mm), marrow diameter (Ma.Dm; mm) and cortical thickness (Ct.Th, µm). Additionally, 6 fields with a 40× magnification objective were also captured from each section of a representative sub sample for quantification of cellular density parameters, namely: osteocyte number (N.Ot; /mm^2), empty lacunae number (N.Lc; /mm^2) and empty lacunae to osteocyte ratio (N.Lc/N.Ot). All histomorphometric parameters were either directly measured or calculated according to the ASBMR guidelines [26].

Skeletal muscle extraction and Citrate Synthase (CS) activity determination

Due to its role as a pace-making enzyme in the first step of the Krebs Cycle, CS activity was used as an indicator of mitochondrial density and aerobic capacity in skeletal muscle, and assayed to confirm the effectiveness of the differential housing conditions between AS and AA animals in the induction of the different physical activity patterns.

Immediately after sacrifice, the right hind limb muscles (*soleus, gastrocnemius, tibialis anterior and quadriceps femoris*) were excised, cleared of surrounding connective tissue and weighed on a precision scale. Briefly, muscles were minced in ice-cold isolation medium containing 100 mM sucrose, 0.1 mM EGTA, 50 mM Tris/HCl, 100 mM KCl, 1 mM KH_2PO_4 and 0.2 % bovine serum albumin (Sigma; St Louis) pH 7.4. Minced blood free tissue was rinsed and suspended in 10 ml of fresh medium containing bacterial proteinase (Nagarse E.C.3.4.21.62, type XXVII; Sigma) at 0.2 mg/ml and 0.1 % Triton X-100 and stirred for 2 min. The sample was then homogenized in a Potter-Elvehjem homogenizer and pestle on ice. After homogenization, 3 volumes of Nagarse-free isolation medium were added to the homogenate. A sample was then collected for biochemical assessment of CS activity and total protein content determination. CS activity was measured according to Coore and colleagues [10] by spectrophotometric (412 nm) measurement of the amount of 5.5-dithiobis (2-nitrobenzoate) that reacted with acetyl-coenzyme A (CoA) upon release from the reaction of acetyl-CoA with oxaloacetate. Total protein concentration was spectrophotometrically determined according to Lowry and colleagues [19], using bovine serum albumin as standard.

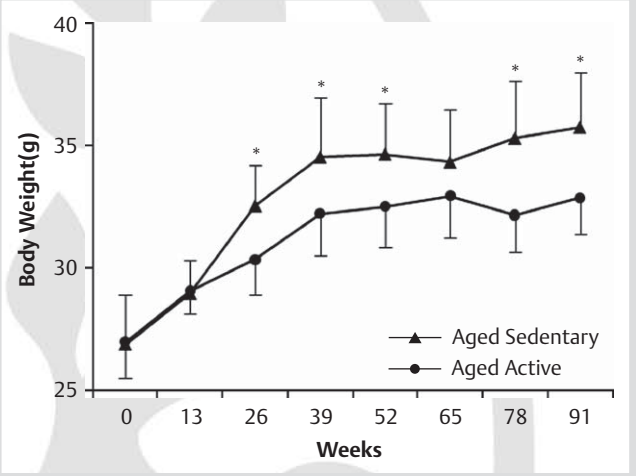

Fig. 2 Average body weight evolution of the aged active (AA) and aged sedentary (AS) groups throughout the 23 months of experimental period. Following week 13 body weight of sedentary animals was consistently higher compared to animals from the active group. * Significant differences between groups ($p < 0.05$).

Statistics

Means and standard deviations were calculated for all variables of the 3 groups. After assaying the variables for normal distribution with the *Kolmogorov-Smirnov* test with *Lilliefors* significance correction, groups were compared for the histomorphometry indices using a model of analysis of covariance (ANCOVA). To eliminate data contamination resulting from the comparison of experimental groups that differ in body weight and size, all comparisons were adjusted to the weight of the animal. To verify the extent to which the bone outcomes were associated with the amount of exercise performed throughout life, *Pearson* correlation coefficient was determined between the histomorphometric variables and the total distance travelled by the animals of the AA group. Independent samples *t*-test was performed to analyze the differences in body weights, skeletal muscle weights and CS activity between the AS and AA groups of animals. Data was analyzed with SPSS 17.0 statistical software and significant differences considered when $p < 0.05$.

Results

Voluntary physical activity pattern and weight

Two animals from the AS group died during the experimental procedure and therefore were not included in the results. During the 3 initial months of the experimental protocol, the animals in the active group registered a mean running distance of 9 km per day, ranging from 6 to 13 daily km (▶ **Fig. 1**). After this initial stage of more pronounced activity, the average daily distance fell to about 6 km a day during the succeeding 7 months, after which it stabilized around 5 km a day until sacrifice. The analysis of the animals' patterns of physical activity shows that they were substantially more active during the initial months of age, having afterwards a decline in the amount of the daily running distance. Despite this decline, animals in this group evidenced a significant predisposition to exercise during the entire duration of the experimental protocol, showing therefore that an active pattern of behaviour was maintained throughout life.

Accordingly, weight evolution also shows that animals housed in cages without running wheel became heavier than animals housed in cages with wheels, suggesting body weight increase as a consequence of sedentary behaviour (▶ **Fig. 2**).

Table 1 Body weight, hind limb skeletal muscle weight and citrate synthase activity.

	Aged sedentary mean ± sd	Aged active mean ± sd
body weight (g)	36.14 ± 3.36[a]	33.09 ± 1.99
hind limb muscle weight (g)	1.76 ± 0.14[a]	1.97 ± 0.16
ratio muscle/body weight (%)	4.87 ± 0.78[a]	5.95 ± 0.51
CS activity (mmol/min/mg)	47.71 ± 5.59[a]	58.24 ± 8.43

CS – citrate synthase. [a] Statistically significant difference *vs.* aged active group ($p < 0.05$). Results are mean ± standard deviation (sd)

Skeletal muscle weight and CS activity

Despite having a significantly higher body weight, animals housed in cages without running wheels (AS) had a significantly smaller hind limb skeletal muscle weight than animals that were allowed to exercise (AA), even after adjusting the comparisons of skeletal muscle weight to total body weight. Determination of hind limb CS activity showed significant differences between AA and AS animals, with a noticeable smaller activity in the AS group (▶ **Table 1**).

Histomorphometry of the proximal epiphysis

When analyzing the selected parameters of bone histomorphometry of the proximal epiphysis, the detrimental effects of age on bone structure were evident, with the identification of a significant decrease in both the thickness of cortical bone (Ct.Th) as well as a decrease in all the parameters associated with the amount of cancellous bone in the AA group (▶ **Fig. 3, 4**). When comparing AA and AS animals, significant differences between these groups were evidenced mainly in variables associated with cancellous bone, since there were no differences identified in cortical thickness. Animals that experienced lifelong sedentary behaviour showed decreased Cn.BS, Cn.BS/TV and Tb.N as well as an increased Tb.Sp when compared to their active counterparts (▶ **Table 2**).

Although comparison between both aged groups revealed differences in several parameters, no significant correlation coefficients were identified between the amount of voluntary activity and the majority of the selected parameters in the AA group. Only trabecular thickness showed to be significantly correlated with the amount of physical activity in this group (▶ **Table 2**).

Histomorphometry of the distal epiphysis

The detrimental effect of age in skeletal structure was also noticeable in the distal epiphysis of the femur, as significant dif-

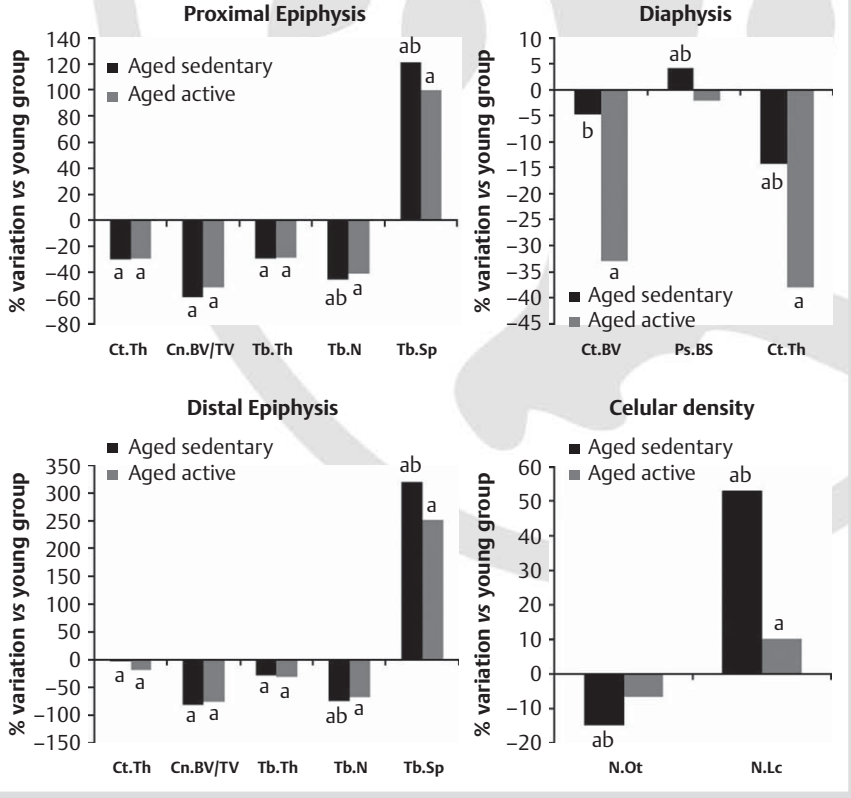

Fig. 3 Percent variation and group comparison of the histomorphometric parameters analyzed in each anatomical region of the femur. Data is expressed in percentage and represents variation relative to young group. Ct.Th = cortical thickness; Cn.BV/TV = cancellous bone volume (relative); Tb.Th = trabecular thickness; Tb.N = trabecular number; Tb.Sp = trabecular separation; Ct.BV = cortical bone volume; Ps.BS = periosteal bone surface; N.Ot = osteocyte number; N.Lc = empty lacunae number. **a** Statistically significant difference vs. young group ($p < 0.05$), **b** Statistically significant difference vs. aged active group ($p < 0.05$).

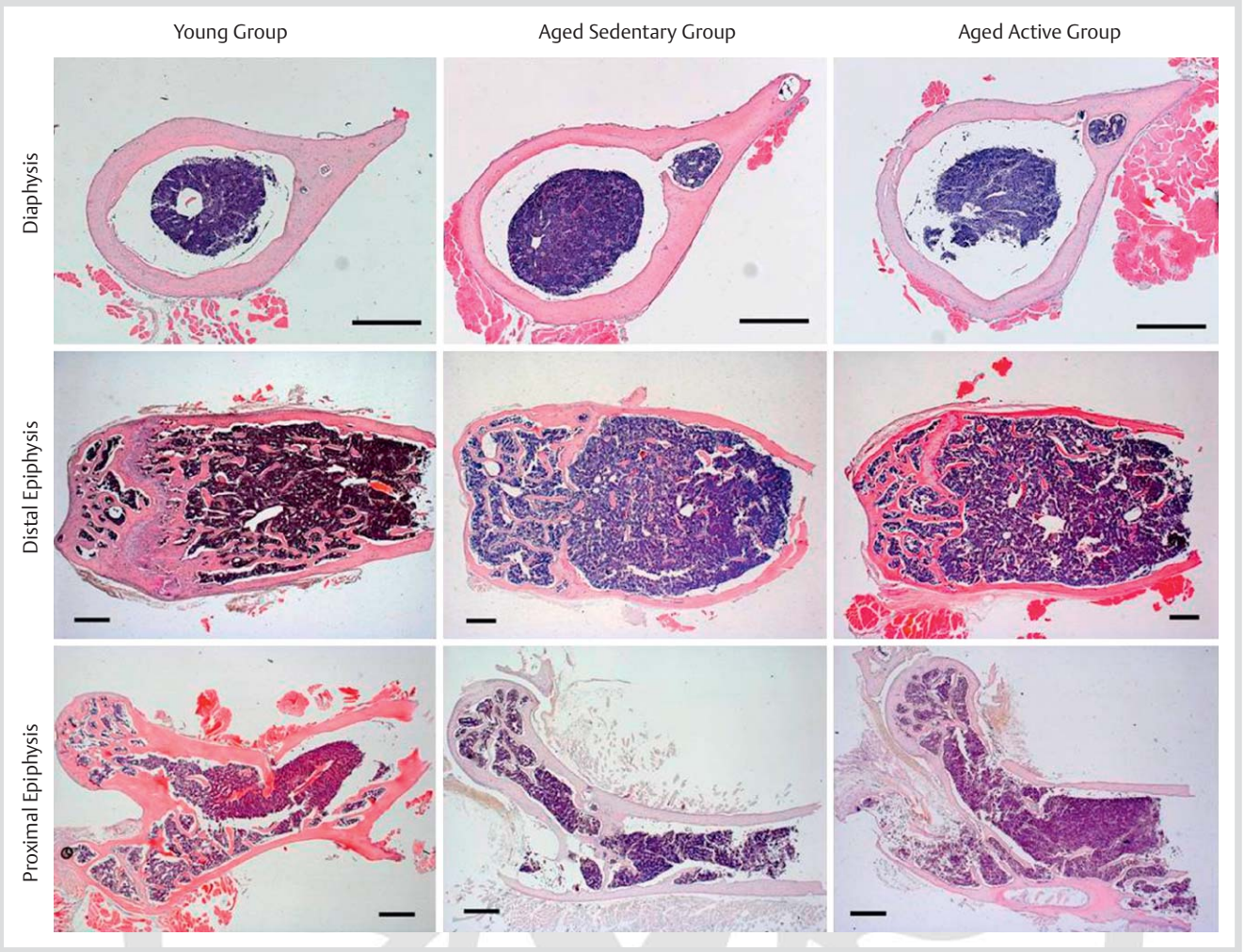

Fig. 4 Representative photomicrographs of analyzed femur anatomical regions from mice of the 3 experimental groups. First row – photomicrographs of mid femoral diaphysis axial sections in each group. Second and third rows – coronal sections of both epiphysis showing differences in trabecular bone microarchitecture between groups. Several age associated hallmarks of bone fragility are shown to be increased by sedentary behaviour and therefore are not age related per se. Sedentary behaviour significantly contributed to the decrease in Tb.N and consequently to the increase in Tb.Sp in both epiphysis. Bars represent 500 µm.

ferences in all parameters were identified between young and both aged groups (**Table 2**; **Fig. 4**). Furthermore, the same variables that revealed significant differences between sedentary and active groups in the proximal epiphysis were also identified in the distal epiphysis, namely Cn.BS, Tb.N, Cn.BS/TV and Tb.Sp. Therefore, the same skeletal pattern was recognized, with sedentary animals having reduced Tb.N and increased Tb.Sp compared to active animals (**Fig. 3**). Contrary to the proximal epiphysis, all the variables related to cancellous bone microarchitecture were here significantly correlated with the amount of activity performed by the active animals. The only variable that did not show correlation with travelled distance was Ct.Th, which also did not show significant differences between both aged groups (**Table 2**).

Histomorphometry of the diaphysis

The histomorphometry parameters of the diaphysis indicate that age significantly influenced this region dimensions, affecting both the cortical bone as well as the medullar cavity dimensions (**Table 2**; **Fig. 4**). In the AS group, animals revealed a larger mid-diaphysis cross-sectional area (B.Dm) when compared to the Y group. This increase was due to a significant enlargement of the marrow cavity (Ma.V) since they evidenced a decrease in the cortical thickness (Ct.Th). Conversely, animals in the AA group showed no significant differences in several variables when compared to Y animals, namely in TV, Ps.BS and B.Dm. Although the cross-sectional area of the diaphysis was unaltered in the AA group compared to the Y group, their medullar cavity was significantly larger, resulting in a significant smaller cortical bone volume (Ct.BV) and cortical thickness (Ct.Th) in AA group compared to Y group (**Fig. 3**). Interestingly, physical activity appeared to be related to a more pronounced decrease of diaphysis cortical thickness, since the differences between AS and Y groups for this variable were smaller than between AA and Y groups. This relationship was also supported by the identification of a significant negative correlation coefficient between the variables cortical thickness and cortical bone volume with the travelled distance by the AA animals (**Table 2**).

Table 2 Histomorphometric analysis of the 3 femur regions in each group and correlation coefficients between histomorphometry and total distance covered by AA animals.

	Young	Aged sedentary	Aged active		
	mean ± sd	mean ± sd	mean ± sd	Running Distance	
				r	p
proximal epiphysis					
Ct.Th (µm)	165.98 ± 31.05	115.80 ± 19.64[a]	117.00 ± 9.54[a]	−0.14	0.330
TV (mm^3)	5.45 ± 0.81	5.85 ± 0.99	6.02 ± 0.87	−0.55	0.704
Cn.BV (mm^3)	1.06 ± 0.32	0.43 ± 0.11[a]	0.51 ± 0.23[a]	0.09	0.549
Cn.BS (mm^2)	38.97 ± 7.08	22.59 ± 5.11[a,b]	26.24 ± 11.27[a]	0.18	0.214
Cn.BV/TV (%)	19.70 ± 6.28	7.38 ± 1.94[a]	8.19 ± 2.46[a]	0.09	0.521
Cn.BS/TV (mm^2/mm^3)	7.17 ± 0.99	3.90 ± 0.98[a,b]	4.24 ± 1.13[a]	0.27	0.059
Tb.Th (µm)	54.17 ± 13.80	38.05 ± 6.28[a]	38.54 ± 4.35[a]	0.39	<0.01
Tb.N (/mm)	3.59 ± 0.50	1.95 ± 0.49[a,b]	2.12 ± 0.57[a]	0.27	0.059
Tb.Sp (mm)	0.23 ± 0.05	0.51 ± 0.16[a,b]	0.46 ± 0.11[a]	−0.25	0.074
distal epiphysis					
Ct.Th (µm)	115.94 ± 25.72	113.23 ± 29.79[a]	95.04 ± 18.72[a]	0.18	0.224
TV (mm^3)	6.52 ± 0.59	6.70 ± 0.89	6.90 ± 0.85	−0.12	0.452
Cn.BV (mm^3)	0.78 ± 0.21	0.15 ± 0.04[a]	0.19 ± 0.05[a]	0.50	<0.01
Cn.BS (mm^2)	46.89 ± 5.78	12.82 ± 2.45[a,b]	16.39 ± 4.41[a]	0.36	0.013
Cn.BV/TV (%)	11.92 ± 3.06	2.27 ± 0.54[a]	2.72 ± 0.57[a]	0.70	<0.01
Cn.BS/TV (mm^2/mm^3)	7.21 ± 0.80	1.91 ± 0.30[a,b]	2.35 ± 0.47[a]	0.58	<0.01
Tb.Th (µm)	32.92 ± 6.86	23.67 ± 4.26[a]	23.01 ± 2.58[a]	0.31	0.033
Tb.N (/mm)	3.60 ± 0.40	0.96 ± 0.15[a,b]	1.17 ± 0.23[a]	0.58	<0.01
Tb.Sp (mm)	0.25 ± 0.03	1.05 ± 0.19[a,b]	0.88 ± 0.21[a]	−0.53	<0.01
diaphysis					
TV (mm^3)	1.76 ± 0.14	2.07 ± 0.25[a,b]	1.75 ± 0.42	−0.32	<0.01
Ct.BV (mm^3)	0.85 ± 0.14	0.81 ± 0.23[b]	0.57 ± 0.20[a]	−0.52	<0.01
Ps.BS (mm^2)	5.54 ± 0.54	5.77 ± 0.45[a,b]	5.42 ± 0.70	−0.34	<0.01
Ma.V (mm^3)	0.92 ± 0.09	1.26 ± 0.20[a,b]	1.19 ± 0.32[a]	−0.08	0.296
Ma.Pm (mm)	3.74 ± 0.30	4.47 ± 0.73[a]	4.38 ± 0.62[a]	−0.13	0.129
B.Dm (mm)	1.50 ± 0.06	1.62 ± 0.11[a,b]	1.48 ± 0.19	−0.32	<0.01
Ma.Dm (mm)	1.08 ± 0.06	1.26 ± 0.11[a,b]	1.21 ± 0.16[a]	−0.14	0.101
Ct.Th (µm)	0.21 ± 0.03	0.18 ± 0.05[a,b]	0.13 ± 0.04[a]	−0.48	<0.01
cellular density					
N.Ot ($/mm^2$)	1 028.80 ± 92.90	876.50 ± 100.70[a,b]	962.30 ± 118.20		
N.Lc ($/mm^2$)	244.10 ± 58.20	373.90 ± 98.80[a,b]	269.00 ± 72.20[a]		
N.Lc/N.Ot	0.241 ± 0.065	0.441 ± 0.160[a,b]	0.286 ± 0.094[a]		
Ot (%)	80.80 ± 4.30	70.20 ± 7.20[a,b]	78.10 ± 5.50[a]		
Lc (%)	19.20 ± 4.30	29.80 ± 7.20[a,b]	21.90 ± 5.50[a]		

Ct.Th = cortical thickness; TV = tissue volume; Cn.BV = cancellous bone volume (absolute); Cn.BS = cancellous bone surface (absolute); Cn.BV/TV = cancellous bone volume (relative); Cn.BS/TV = cancellous bone surface (relative); Tb.Th = trabecular thickness (Tb.Th = 2/(BS/BV)); Tb.N = trabecular number (Tb.N = (BV/TV)/Tb.Th); Tb.Sp = trabecular separation (Tb.Sp = (1/Tb.N)-Tb.Th); Ct.BV = cortical bone volume; Ps.BS = periosteal bone surface; Ma.V = marrow volume; Ma.Pm = marrow perimeter; B.Dm = bone diameter; Ma.Dm = marrow diameter; N.Ot = osteocyte number; N.Lc = empty lacunae number; N.Lc/N.Ot = empty lacunae to osteocyte ratio; Ot = osteocyte; Lc = empty Lacuna. The *p*-value in the right column refers to the correlation coefficient between histomorphometric variables and total distance travelled by animals in the AA group. [a] Statistically significant difference *vs*. young group ($p < 0.05$)

[b] Statistically significant difference *vs*. aged active group ($p < 0.05$). Results are mean ± standard deviation (sd)

Cellular density patterns

The analysis of osteocyte (N.Ot) and empty lacunae (N.Lc) distribution pattern in the animals of each group shows that with advancing age, there was a decrease in osteocyte number and a parallel increase in empty lacunae per area, because of the significant differences encountered between AS group and Y group. However, sedentary animals demonstrated these alterations to a much greater extent than their active counterparts (**Table 2**; **Fig. 3**). While animals from the AS group showed significant differences both in osteocyte and empty lacunae number when compared to Y animals, AA mice showed only significant differences in empty lacunae number, these however being of lesser extent than those exhibited by the sedentary group (**Fig. 5**).

Discussion

Our results show that ageing is not the sole cause responsible for the reduction of bone quality observed in the elderly, as sedentary behaviour significantly exacerbates that loss. As expected, we identified in both aged groups a significant reduction of several parameters of cancellous bone distribution when compared to the young group. These differences were however significantly more pronounced in animals confined to lifelong sedentary behaviour. The amount of decrease in voluntary physical activity also showed to be related to the decrease in bone quality as a significant correlation was identified between distance travelled by AA animals and several parameters of cancellous bone distribution. Differences in cellular density patterns were also identified between the 3 groups of animals, with lifelong sedentary animals revealing reduced osteocyte numbers and higher empty

Fig. 5 Details of mid diaphysis axial sections showing osteocyte and empty lacunae distribution in cortical bone from mice of the 3 experimental groups. It is noticeable a significantly higher number of empty lacunae in AS animals compared to both AA and Y animals suggesting that physical inactivity increased osteocyte loss within bone matrix. Magnification is 40×. Bars represent 50 µm.

lacunae number, showing therefore increased signs of bone devitalization.

By monitoring daily physical activity, we identified that mice tended to be active, and to voluntarily spend a significant amount of time running. This need for activity was particularly evident in the first months of the experimental protocol, after which a slight decrease was registered, eventually stabilizing over time until the end of the protocol. This inverse relationship between age and physical activity has also been identified in several other human and non human studies, suggesting that a strong biological component influences it [15]. These findings also demonstrate that experimental animals have a need to be physically active and consequently experimental models restricted to regular cages are not adequate controls of the biological ageing process itself as they also suffer the harmful effects of imposed sedentary behaviour. As a result what we see in animals housed in cages with access to running wheel is a consequence of the ageing process. Conversely, the lifelong changes that occur in sedentary animals are not only the result of ageing but the combined result of ageing together with lifelong sedentary behaviour. Consequently, by comparing AS with AA animals we isolated the effect of sedentary behaviour from the effect of ageing allowing us to determine to what extent age is the major cause for the skeletal fragility observed in the elderly.

The sedentary lifestyle created by the regular housing conditions was evident when AA and AS body weights and hind limb skeletal muscle characteristics were compared. During the second trimester of the experimental procedure AS mice began to significantly increase weight when compared to AA mice (**Fig. 2**) showing progressive signs of obesity. Moreover, AS animals revealed at time of sacrifice extensive atrophy of the hind limb muscle as it was evident from their smaller muscular weight. Both increased weight and muscle atrophy are characteristic features of a sedentary lifestyle, which confirms our animal models for the 2 patterns of behaviour. CS activity determination also corroborates that standard housing conditions were responsible for reducing the animals' movements which lead to an imposed sedentary lifestyle with consequences at skeletal muscle aerobic capacity level.

In our study, we identified several deleterious effects that ageing has on the skeletal structure of the femur by comparing several histomorphometric parameters between AA and Y animals, at 3 different femur sites. In both epiphyses, the amount of cancellous bone was significantly smaller in the AA animals. This decrease in cancellous bone volume (Cn.BV/TV) was a result of both a decrease in trabecular number (Tb.N) and trabecular thickness (Tb.Th). The issue about the age related changes in trabecular thickness is still a source of controversy in the literature as several authors have identified conflicting variations of trabecular thickness with the increase of age [33]. While some identify a global decrease in cancellous bone with age [8], others state that this reduction is mostly due to the number of horizontal trabeculae whereas vertical ones tend to be better preserved over time. The overall decrease in cancellous bone volume that we have registered in our study, had a marked effect in the increase of trabecular separation (Tb.Sp), which is a fundamental property of the 3-dimensional cancellous bone network, and a determinant for maintenance of bone strength [30].

Interestingly, the cancellous bone volume differences that we identified between both aged groups and the young group were more pronounced in the distal epiphysis. Also, at this site, the total decrease in cancellous bone volume was mostly due to the reduction in trabecular number, rather than in thickness, leading to an even greater increase of trabecular separation than in the proximal epiphysis. The observed differences between proximal and distal epiphysis suggest that the underlying mechanisms that lead to the reduction of cancellous bone are site specific. While in some sites cancellous bone is probably eroded evenly over time, in other sites the erosion is uneven, leading to more extensive erosion of some trabeculae, while others may have reduced resorption rates due to their compensatory mechanical role. This might explain the different results described in the literature about the changes in trabecular thickness with age mentioned above.

Despite the fact that both aged groups showed a decrease in cancellous bone parameters compared to young animals, lifelong sedentary behaviour had an exacerbation effect on the encountered differences, as several variables were significantly different between sedentary and active animals. The most noteworthy effect of sedentary behaviour on cancellous bone was the smaller trabecular number compared to active animals. Although there were no differences in cancellous bone volume, sedentary animals had less individual trabeculae which led to a significantly higher trabecular separation. The evidence that the reduction in spontaneous physical activity is a key aspect to the age derived skeletal differences, comes also from the identification of a significant correlation between several cancellous bone parameters and the amount of activity that the animals performed during

the experimental procedure. While in the proximal epiphysis only trabecular thickness showed a significant correlation with the amount of exercise, in the distal epiphysis almost all selected variables were significantly correlated. Once more there seem to be differences between femoral sites regarding the way they are affected by age and by physical activity.

Regarding the cortical bone from mid-diaphysis, several age derived features that have been previously identified in other studies were here particularly evident in sedentary animals [11]. Aged sedentary (AS) animals showed a significantly higher bone diameter (B.Dm) as a result of the enlargement of both periosteal bone surface (Ps.BS) and marrow perimeter (Ma.Pm). This global increase of the cross-sectional dimensions reduces the differences of cortical bone volume (Ct.BV) between sedentary (AS) and young animals despite the marked decrease in cortical thickness in the AS group. Animals from the active group (AA) showed no increase in periosteal bone surface, which is a distinctive feature of aged bones. Additionally, although they revealed an increase in marrow volume (Ma.V), this was less pronounced than in the sedentary group. Interestingly, sedentary animals displayed a less pronounced reduction in cortical thickness than their active counterparts.

We believe that the differences in Ct.Th between sedentary and active animals are explained by the reduction in bone resorption in AS animals resulting in the accumulation of more bone tissue, but at the expense of reducing the removal of older and more brittle bone. When looking at the cellular density patterns in each group of animals, a significant decrease in osteocyte density was identified in sedentary animals. Additionally, the presence of empty lacunae, the histological footprint of osteocyte death, showed here a significant increase. We hypothesize that this noticeable devitalization in sedentary animals could hinder osteoclast activation and the resorption of older bone, since there is evidence both in vitro [31] and in vivo [32] suggesting that the integrity of the osteocyte network is a requirement for bone resorption to occur. We therefore believe that the increased cortical thickness identified in aged sedentary animals does not represent a true net gain due to bone formation, but instead might be the result of a decrease in osteoclastic bone resorption with a consequent accumulation of older bone as a result of reduced osteocyte signalling. Previous studies [36] have also shown a significant correlation between the decrease of osteocyte number and the accumulation of microcracks in cortical bone, suggesting that a viable osteocyte network is necessary for detection and removal of damaged bone.

The differences identified between groups regarding cellular density distribution suggest that age alone does not significantly diminish osteocyte numbers, since aged active animals showed no decrease in osteocyte number compared to young animals. In contrast, aged sedentary animals, displayed significantly less osteocyte numbers compared to their active counterparts. Therefore previous findings suggesting an age-related decrease in osteocyte numbers [27,36], did in fact not determine the effect of age *per se*, but the cumulative effects of age and lack of physical activity. Sedentary behaviour was also shown to significantly increase the presence of empty lacunae, since aged active animals had significantly less empty lacunae than sedentary animals.

Several studies have shown that osteocyte survival relies on a great extent to the mechanical forces that are applied to the bone matrix, with bone unloading increasing osteocyte apoptosis while mechanical stimulation decreases apoptosis [2,3]. Considering therefore the importance of mechanical stimulation for osteocyte viability, it is understandable why sedentary behaviour considerably worsened the effects of age on osteocyte number.

It is also important to mention that bone mass in the elderly is the result of both bone gains during growth and of subsequent bone losses during ageing. Therefore, the differences identified between AA and AS animals at sacrifice might result not only from differences in bone loss rate during adulthood but also from differences in bone mass accrual during growth, especially because this was the age period when AA animals were most active (**Fig. 1**). In addition, although being aware that the use of decalcified paraffin embedded bone sections for histomorphometric analysis might introduce bias to the bone structure, we believe that this limitation was overcome by the use of a standard protocol for the preparation of all samples. The effects resulting from tissue processing were therefore uniform between different samples allowing valid comparisons to be made among groups.

In conclusion, considering our main objective of determining if age is the sole major cause for the skeletal fragility observed in the elderly, or if a decrease in physical activity is also responsible, we were able to show that aged sedentary mice have a more pronounced decrease in several histomorphometric parameters of cancellous bone microarchitecture, as well as significant differences in osteocyte distribution pattern when compared to aged active animals, showing extensive signs of devitalization. The differences identified between AS and AA groups demonstrate that age is not the only responsible factor for the bone fragility identified in the elderly as sedentary behaviour clearly aggravates these differences. It is also our believe that studies that aim to describe age related physiological or structural changes should be sensitive to the fact that a significant amount of these changes are due to the sedentary behaviour induced by typical housing conditions and not only attributable to the ageing process *per se*.

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Study 3

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Abstract: Bone strength depends on several material and structural properties, but findings concerning the best predictors of bone mechanical performance are conflicting. The aim of this study was to investigate how a broad set of bone properties in the proximal femur are influenced by age and hormonal status and how altogether these properties determine bone strength. Twenty-five Wistar rats were ovariectomized (OVX, n=13) or sham operated (SHAM, n=12) at 5 months of age and later sacrificed following 9 months. Another group of rats with 5 months was sacrificed as baseline control (BSL, n=7). At sacrifice, serum 17β-estradiol and bone turnover markers concentration were determined in the serum. Both femurs were collected for assessment of trabecular microarchitecture, femoral neck geometry, radiographic absorptiometry, calcium and phosphate content and biomechanical properties. While stiffness was mostly associated with proximal femur trabecular microarchitecture and mineralization degree, bone strength was mostly linked to bone size and femoral neck geometry which predicted almost 50% of its variance. Despite the decrease in cortical and trabecular bone as well as in mineralization degree following estrogen loss, bone strength was not reduced in OVX animals compared to BSL or SHAM. This was due to a change in femoral neck geometry as well as to an increase in femur size in OVX, which apparently compensated their lower bone volume and mineral content, thereby preserving bone strength. Estrogen loss leads to a deterioration of bone tissue quality but bone strength was preserved at the expense of geometric adaptations.

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Title

Changes in proximal femur bone properties following ovariectomy and their association with resistance to fracture

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Abstract

Bone strength depends on several material and structural properties, but findings concerning the best predictors of bone mechanical performance are conflicting. The aim of this study was to investigate how a broad set of bone properties in the proximal femur are influenced by age and hormonal status and how altogether these properties determine bone strength. Twenty-five Wistar rats were ovariectomized (OVX, n=13) or sham operated (SHAM, n=12) at 5 months of age and later sacrificed following 9 months. Another group of rats with 5 months was sacrificed as baseline control (BSL, n=7). At sacrifice, serum 17β-estradiol and bone turnover markers concentration were determined in the serum. Both femurs were collected for assessment of trabecular microarchitecture, femoral neck geometry, radiographic absorptiometry, calcium and phosphate content and biomechanical properties. While stiffness was mostly associated with proximal femur trabecular microarchitecture and mineralization degree, bone strength was mostly linked to bone size and femoral neck geometry which predicted almost 50% of its variance. Despite the decrease in cortical and trabecular bone as well as in mineralization degree following estrogen loss, bone strength was not reduced in OVX animals compared to BSL or SHAM. This was due to a change in femoral neck geometry as well as to an increase in femur size in OVX, which apparently compensated their lower bone volume and mineral content, thereby preserving bone strength. Estrogen loss leads to a deterioration of bone tissue quality but bone strength was preserved at the expense of geometric adaptations.

Keywords

Osteoporosis

Biomechanical testing

Femoral neck geometry

Bone mineral density

Histomorphometry

Introduction

Osteoporosis is a largely prevalent metabolic bone disease in the elder population as well as in postmenopausal women, being characterized by a generalized loss of bone mass and architectural deterioration. The clinical significance of osteoporosis arises from the increased risk of fracture occurrence following minimal trauma, resulting in the substantial increase of bone fractures in the population above 45 years of age. Fragility fractures are an important health concern as they significantly increase morbidity, mortality risk and are a huge economic burden to the society due to health care costs associated with medical treatment and reduced productivity. One of the major challenges of osteoporosis is that it remains clinically silent until it manifests as a fracture, making therefore essential the establishment of adequate screening strategies that allow reliable, timely and cost-effective detection of those at risk of fracture.

Currently, DXA derived measures of areal bone mineral density (aBMD) are the gold-standard for diagnosing osteoporosis and for the clinical assessment of fracture risk. However, regardless of epidemiological evidence that aBMD correlates with fracture risk [1] it is only able to predict a portion of the individuals fracture risk [2], which leads to insufficient recognition of those that are more likely to experience a fracture [3]. For instance, reports show that 66% and 79% of non-vertebral fractures occur in women and men without diagnosed osteoporosis, respectively [4]. Additionally, age related decreases in femur strength are steeper than decreases in aBMD [5], suggesting that the population at risk for fragility fractures is underestimated according to the DXA diagnosis criteria, which has led to the discouragement of osteoporosis screening programs relying only in aBMD as an endpoint [1]. Despite the clinical relevance of aBMD and of low bone mass as a component of fracture risk, it is now well established that bone strength is influenced by a set of orchestrated features [6]. Skeletal fragility is therefore a complex entity and the final outcome of the interaction

between several material and structural bone properties [7]. Hence, the use of aBMD alone for estimating fracture risk is largely insufficient and likely misleading as it provides limited information about bone tissue material composition and structural design [7].

To improve the assessment of fracture risk, efforts have been made to determine to what extent, other than aBMD bone features, such as bone geometry [8], microarchitecture [9] or bone turnover [10] might influence bone strength as well as to determine how these features are affected by age or hormone status thereby contributing to bone fragility. Nevertheless, evidence showing their association with fracture risk has been provided by different studies that independently determine how variations in these properties affect bone resistance. Conflicting results [11, 12] have however complicated the assessment of the contribution of each of these separate bone features to bone strength. Additionally, as some bone features might adapt in order to compensate for deficits in others [13, 14], the assessment of isolated bone features makes difficult to determine how changes in one property might influence the others and thereby bone strength. For instance, it has been previously described that with age there is an increase in femoral neck section modulus, which biomechanically compensates for decreases in bone mass [13]. A similar adaptive response has also been described in the ulna [14]. Therefore, as none of the determinants of bone strength alone appears to be able to express the bone´s mechanical competence, a comprehensive assessment of how several of these properties are influenced by age and hormonal status can provide useful insights into how bone adapts itself to changes in some features and how they, altogether, determine the strength of the bone, thereby leading to bone fragility.

The objective of our study was then to investigate how bone mineral content, trabecular microarchitecture, femoral neck geometry, radiographic absorptiometry (RA) and biochemical markers of bone turnover are influenced by estrogen loss in the ovariectomized (OVX) rat model and how well are they associated with the proximal femur biomechanical properties.

Materials and methods

Animal models and experimental design

Following arrival, 32 nulliparous female Wistar rats with 5 months old (Charles River Laboratories, Barcelona, Spain) were randomly ovariectomized (OVX; n=13), sham operated (SHAM; n=12) or sacrificed as baseline controls (BSL; n=7) following one week of quarantine. Bilateral ovariectomy was performed by standard ventral approach under anesthesia with 4% sevoflurane. In SHAM animals the ovaries were only exposed but not removed. OVX and SHAM animals were maintained for 9 months in an inverted 12h light/dark cycle in a humidity/temperature controlled environment. Standard rat chow (A04, SAFE, Augy, France) and water were provided *ad libitum* throughout the experimental period. Body weight of each animal was recorded weekly with a precision balance (Kern 440, Balingen, Germany). All procedures involving animal care were approved by the local ethics committee (University of Porto).

Animal sacrifice and tissue harvesting

At 14 months of age, OVX and SHAM animals were sacrificed by exsanguination under anesthesia with 4% sevoflurane. Serum was separated from the blood by centrifugation at 1500rpm for 30min at 4^{o}C and used for assaying estrogen and markers of bone turnover concentration. Both femurs were removed and cleared of surrounding soft tissue. Right femur was used for assaying femoral neck geometry, RA, biomechanical testing and quantification of calcium and phosphate content. Left femur was used for assaying trabecular microarchitecture.

Biochemistry

Biochemical parameters were assayed in the serum using commercially available ELISA kits and a spectrophotometer (iEMS Labsystems, Washington, USA) according to the manufacturer recommendations. 17β-estradiol concentration was assayed with Estradiol DE2693 ELISA kit (Demeditec Diagnostics, Kiel-Wellsee, Germany) for the confirmation of the OVX animal model. Assay range was between 9.7 and 2000pg/mL. Inter and intra-assay coefficients of variation (CV) are lower than 9.4% and 6.8%, respectively. Bone formation was assayed by measuring osteocalcin (OC) concentration with Rat-MID™ ELISA kit (Immunodiagnostic Systems, Boldon, UK). Assay range was between 50 and 1500ng/mL. Inter and intra-assay CV are lower than 7.7% and 5.0%, respectively. Bone resorption was assayed by measuring C-terminal telopeptides of type I collagen (CTX) concentration with RatLaps™ ELISA kit (Immunodiagnostic Systems, Boldon, UK). Assay range was between 7.7 and 188ng/mL. Inter and intra-assay CV are lower than 14.8% and 9.2% respectively. Positive controls for OC and CTX were assayed together with samples and their concentration was found to be within the manufacturer's quality control concentration limits.

Radiographs

Digital radiographs were taken from the right femur in the craniocaudal direction using dental radiographic equipment (CCX Digital, Trophy, Croissy-Beaubourg, France). The right femur was positioned over the sensor plate (DenOptix Imaging Plates, Sterling Heights, USA) at a 20cm distance from the X-ray-source. Exposure time and intensity were 0.08sec at 70Kv and 8mA. To ensure equal positioning between samples, all bones were placed with the caudal surfaces of both condyles and of the trocanter minor in contact with the film. A standard referent made from a cortical bone section with 10mm of length was included in each radiograph for calibration purposes.

Radiographic absorptiometry

Bone mass was assayed in the right femur by RA as described previously [15] using imageJ software (NIH, Bethesda, USA). Correlation between RA and DXA derived aBMD is shown to be as good as between different DXA scanners themselves [16]. Briefly, each x-ray image was composed of pixels within a grayscale value between 0 and 255, were 0 is black and 255 is white. Whiter pixels correspond to higher radiopacity. Bone mass was determined in each region as the mean gray value (MGV) of the pixels within that region. For total bone mass, the boundary of the femur was manually traced and the whole bone defined as the region of interest (ROI). For the other analyzed regions, including the standard referent, a circular selection with 1.5mm of diameter was defined as the ROI and positioned according to anatomical landmarks. The MGV registered for each region was the average of 4 repeated measures. In order to consider differences in background noise, the MGV of the background in the vicinity of the ROI was subtracted to the MGV of each region analyzed. Results were expressed as percentage of the MGV of the standard referent. The regions analyzed were total femur, femoral head, upper and central area of the femoral neck, trocanter major and minor and intertrochanteric region (Fig.1a). The CVs between repeated measures were 0.05%, 0.26%, 1.32%, 0.17%, 1.45%, 1.50% and 1.59%, respectively.

Femoral neck geometry

Femoral neck geometry was assayed on the digital radiographs of the right femur by measuring standard geometric parameters using ImageJ software as described previously [17] (Fig. 1b). Briefly, femoral-neck axis length (FNAL) was the distance from the lateral aspect of the trocanter major to the medial surface of the femoral head. Femoral neck-shaft angle (NSA) was the angle between the femoral neck and shaft axes. Femoral-neck width (FNW) was

measured at the narrowest region perpendicular to the femoral neck axis. Compression force moment arm (CFMA) was the distance between femur axis and the medial surface of the femoral head. Results were the average of 2 repeated measures. Relative lengths were determined and expressed as percentage of femur length in order to allow comparisons independently of femur length. CVs between repeated measures were found to be among 0.07% (femur length) and 2.8% (CFMA).

Histomorphometry

Left femur proximal third was sectioned with a circular diamond saw continuously wet in could PBS (pH 7.2) and fixed and decalcified in a solution containing 4% paraformaldehyde, 2.5% sucrose (Sigma, St. Louis, USA), 0.1% gluteraldehyde (TAAB, Aldermaston, UK) and 10% EDTA (Sigma) in PBS (pH 7.2) at 4^{o}C for 3 weeks. It was then dehydrated through graded ethanol (Panreac, Barcelona, Spain) concentrations and mounted in paraffin (MERK, Darmstadt, Germany). Six-µm-thick sections were obtained from the head and neck region in the coronal plane, stained with H&E and viewed under a light microscope coupled to a digital camera (Axio Imager A1, Carl Zeiss, Oberkochen, Germany). Images were analyzed with imageJ. Standard histomorphometric parameters were determined as the average of five sections per specimen and included trabecular bone volume fraction (BV/TV, %), trabecular number (Tb.N; /mm^2), trabecular separation (Tb.Sp, µm), trabecular thickness (Tb.Th, µm) and cortical thickness (Ct.Th, µm). Trabecular architecture and Ct.Th were determined in the region between the femoral head growth plate and the lesser trocanter.

Biomechanical testing of the femoral neck

Biomechanical properties were assayed by compression testing of the right femur head according to recommend guidelines [18] using a servo-hydraulic testing machine (TIRATest

2705, Schalkau, Germany). Each femur midpoint was determined and sectioned with a circular diamond saw. The proximal half was then fixed on the testing apparatus and a vertical load applied on the femoral head parallel to the diaphysis. A stabilizing preload was first applied at a rate of 0.1mm.s^{-1} until 5N was reached. Above this point the load rate shifted to 0.5mm.s^{-1} until failure. Bones were kept moist in PBS throughout the testing procedure. The load-deformation plot and bone ultimate strength (maximal load registered, N) were obtained directly from the test and converted to stress-strain. Ultimate stress was the maximum stress the bone could sustain and ultimate strain was the strain at failure. Young´s modulus was the highest slope of the stress-strain curve. Energy to fracture was the area under the load-deformation curve, expressed in mega Joule (MJ). CV for compression testing was 7.6%.

Bone mineral content and degree of mineralization

Bone mineral content was determined by quantification of the calcium and phosphate extracted during the decalcification of the right femur, as described elsewhere [19, 20]. Briefly, after compression testing, the proximal and distal halves of the right femur were collectively fixed overnight in 70% ethanol (Panreac), lyophilized during 4 days (EZ550Q, FTS Systems, Warminster, USA) and their dry weight determined with an analytical balance (Kern 870, Balingen, Germany). They were then decalcified in 5mL of 2% formic acid (Sigma) during 10 days. In each day a 2mL aliquot of the decalcification solution was collected, the remaining discarded and replaced by 5mL of fresh solution. Calcium content was then determined as the sum of the calcium in each daily aliquot by atomic absorption spectroscopy (Perkin Elmer AAnalyst 100, Massachusetts, USA) after addition of 0.5% lanthanum chloride (Sigma). Degree of mineralization was the ratio between calcium content and both halves dry weight. Calibration solutions (Sigma) were read between sets of samples to minimize effects of equipment drift. Recovery degree was found to be on average 99.1% and CV between

repeated samples to be 2.84%. Phosphate content was determined by spectrophotometry (iEMS, Labsystems) based on the formation of molybdate complex using KH_2PO_4 solutions as standard (Sigma). Total phosphate content and density were calculated as described above for calcium. Average CV between repeated samples was 3.55%.

Statistical analysis

The Kolmogorov-Smirnov and Levene´s test were used to investigate within-group normality and homogeneity of variance of each variable, respectively.

Comparisons between groups were performed by one-way-ANOVA with Bonferroni or Games-Howell *post hoc* test whenever homogeneity of variance was verified or not, respectively. When normal distribution was not verified, natural log transformation was employed to reestablish the necessary assumptions for ANOVA. Correlation analysis was performed using Pearson correlation coefficient, except for Energy to fracture in which Spearman's Rho was employed due to lack of data normal distribution. Linear and simultaneous multiple regression analysis were conducted to investigate the best predictors of femoral neck biomechanical properties. Results are expressed as mean ± standard deviation and differences were considered significant when $p<0.05$. Data was analyzed with PASW 18.0 (IBM, Armonk, USA).

Results

Means and standard deviations for each variable assayed are listed in Table 1. Quantification of serum 17β-estradiol concentration revealed the expected differences between OVX and both SHAM and BSL groups, confirming low estrogen levels in OVX animals.

The animal's initial body weight was 262±16.5g, 264±22.9g and 256±19.5g for the BSL, OVX and SHAM groups, respectively (Fig 2). During the subsequent 9 months, body weight increases were significantly higher in OVX compared to SHAM animals ($p<0.01$), resulting in a significantly higher body weight at the time of sacrifice in OVX animals (384±38.3 *vs.* 313±40.2, $p<0.01$).

Histomorphometric analysis of the femoral neck trabecular microarchitecture showed that in OVX animals there was a significantly lower BV/TV and Tb.N compared to both BSL and age matched (SHAM) controls, which was accompanied by a correspondent increase in Tb.Sp. The average Ct.Th in OVX animal's proximal femur was also significantly lower compared to SHAM.

Direct quantification of bone mineral content by atomic absorption spectroscopy showed that SHAM and OVX animals had similar total calcium and phosphate contents, which in both groups was significantly higher than in BSL. However, after normalizing bone mineral content to tissue dry weight, a significantly lower degree of mineralization was identified in OVX animals compared to SHAM and BSL. Calcium to phosphate ratio was identical throughout all groups.

Despite the differences between OVX and SHAM animals regarding both the degree of mineralization and the amount of trabecular (BV/TV) and cortical bone (Ct.Th), no differences were identified between OVX and SHAM groups for RA measurements at all sites analyzed, with the exception of the femoral head in which it was found to be significantly lower in OVX compared to SHAM animals.

Bone resorption was increased in OVX animals as evidenced by their significantly higher concentration in CTX compared to both SHAM ($p<0.01$) and BSL ($p=0.05$) groups. Bone formation in turn was not shown to parallel the OVX animals higher bone resorption, as there were no significant differences between OVX and both SHAM and BSL animals regarding OC concentration ($p=0.130$ and $p=0.330$, respectively). CTX was inversely correlated with both calcium density ($r=-0.511$, $p<0.01$) and phosphate density ($r=-0.427$, $p<0.05$).

There was an increase in bone size with age, evidenced by the higher femur length in both OVX and SHAM groups compared to BSL. However, in addition to age, estrogen concentration also influenced femur geometry. Femur length was higher in OVX compared to SHAM and BSL ($p<0.01$). Moreover, NSA ($p<0.05$) and FNAL, both in absolute terms ($p<0.01$) and after normalization to bone length (relative FNAL, $p<0.05$) were shown to be significantly higher in OVX animals compared to SHAM. FNAL was significantly correlated with calcium density ($r=-0.470$, $p<0.01$), BV/TV ($r=-0.704$, $p<0.01$), Tb.N ($r=-0.578$, $p<0.01$), Tb.Sp ($r=0.618$, $p<0.01$), and whole femur RA ($r=-0.527$, $p<0.01$).

Compression testing revealed that there were no significant differences in the femoral neck biomechanical properties between groups, with the exception of ultimate strain which was significantly lower in SHAM compared to BSL ($p<0.05$). However, when biomechanical performance was expressed relative to calcium density (Fig. 3) OVX animals achieved on average a higher ultimate stress than both BSL and SHAM animals. Significantly higher ultimate stress, ultimate strain and Young´s modulus were also observed in OVX animals compared to both BSL and SHAM when these were normalized to BV/TV. No differences were identified when biomechanical performance was normalized to geometric properties.

To identify the degree of association between the several bone properties assayed (trabecular bone architecture, mineral content, bone geometry, biochemical markers of bone turnover and RA measurements) and the biomechanical performance of the femur, correlation

analysis was performed (Table 2). Ultimate strength correlated significantly with femur length (r=0.470, p<0.05), FNW (r=0.504, p<0.01) and FNAL (r=0.559, p<0.01). Importantly, these parameters were still correlated with ultimate strength after performing partial correlations controlling for BV/TV (r=0.414, p<0.05; r=0.445, p<0.05 and r=0.430, p<0.05, respectively), calcium content (r=0.473, p<0.05; r=0.502, p<0.01 and r=0.581, p<0.01, respectively) and calcium density (r=0.431, p<0.05; r=0.475, p<0.01 and r=0.442, p<0.05, respectively), showing that they were significantly correlated with bone strength independently of the amount of bone tissue and of its degree of mineralization.

Multiple regression analysis was performed to investigate the extent to which proximal femur geometric properties predicted ultimate strength. Simultaneous combination of FNAL, CFMA, FNW and bone length in the multiple regression analysis described 47.1% of the ultimate strength variance (adjusted R^2=0.471, p<0.01). All the remaining variables were unable to significantly predict ultimate strength variation.

Young´s modulus correlated significantly with both Tb.Th (r=0.502, p<0.01), calcium content (r=0.449, p<0.01) and phosphate content (r=0.389, p<0.05). Tb.Th correlated with young´s modulus even after controlling for BV/TV (r=0.535, p<0.01) and calcium density (r=0.507, p<0.01). Multiple regression analysis showed that 31.4% of Young´s modulus variance was predicted by the combination of the variables Tb.Th, calcium content and phosphate content (adjusted R^2=0.314, p<0.01).

Ultimate strain correlated inversely with both femoral neck Ct.Th (r=-0.497, p<0.01) and mineralization degree (*vs* calcium density, r=-0.521, p<0.01; *vs* phosphate density, r=-0.359, p<0.05) as well as positively with OC (r=0.406, p<0.05) and CTX (r=0.511, p<0.01). 42.3% of ultimate strain variation was predicted by Ct.Th, calcium density, phosphate density, OC and CTX concentration (adjusted R^2=0.423, p<0.01).

Energy to fracture was also inversely correlated with both Ct.Th (r=-0.385, p<0.05) and calcium content (r=-0.356, p<0.05). However, multiple regression analysis showed that none of the variables assayed was able to significantly predict its variance.

RA measurements were not shown to correlate significantly with any of the outcomes of biomechanical performance. Correlation analysis showed that all RA measurements were significantly correlated with calcium content (0.406<r<0.578, 0.002<*P*<0.044), with the exception of the femoral head (r=0.338, *p*=0.098). However, multiple regression analysis demonstrated that calcium content and calcium density were only able to explain 29.3% (adjusted R^2=0.293; p<0.01) of the total femur RA variation while BV/TV and Tb.N were shown to explain 31.8% (adjusted R^2=0.318, p<0.01) of the total femur RA variation. When included together in the multiple regression model these variables explained 55.4% (adjusted R^2=0.554; p<0.001) of total femur RA variation, showing that RA was dependent on both the amount and on the degree of bone tissue mineralization and that both contribute significantly to its variation. Besides correlating positively with both the amount and mineralization degree of bone tissue, RA at the femoral head (r=-0.459, p<0.01), femoral neck (r=-0.396, p<0.05), trochanter major (r=-0.473, p<0.01), trochanter minor (r=-0.422, p<0.05), and total femur correlated inversely with FNAL.

Discussion

Our results show that the loss of estrogen in OVX animals led to a significant decrease in the amount of trabecular bone tissue in the proximal femur as well as in the degree of bone tissue mineralization. Despite the importance of these properties to the femoral neck biomechanical performance, as evidenced by their significant correlation with Young´s modulus, we found that bone strength was not significantly reduced in OVX animals compared to both BSL and SHAM. Our results also show that femoral neck geometry was significantly affected by the loss of ovarian hormones, with OVX animals showing a higher femur length, femoral neck axis length and neck shaft angle. Furthermore, these geometric properties were shown to correlate significantly with bone strength and to predict almost 50% of its variance, therefore contributing significantly to the preservation of the femoral neck biomechanical performance in OVX animals counteracting the effects of reduced bone volume and tissue mineralization in bone strength. RA measurements however were not shown to be significant predictors of the femoral neck biomechanical performance.

As expected, ovariectomy resulted in a significant loss of bone tissue, which was due to both the loss of trabecular bone volume as well as to a reduction in the cortical bone shell thickness in the proximal femur region. As it is well established by the estrogen-centric model of osteoporosis [21], this decrease in bone tissue is the result of the increased bone turnover and of the unbalance between bone formation and resorption that follows estrogen loss. This unbalance and the increase in bone turnover following estrogen loss was also identified in our study through the quantification of CTX and OC, which showed mean higher serum concentration in OVX (though not reaching statistical significance for OC; p=0.130) compared to age matched controls.

It is well established that estrogen exerts a pivotal role on the activity of bone cells, namely by inhibiting the differentiation and by promoting the apoptosis of osteoclasts [22] as well as

by inhibiting the renewal of early osteoblast progenitors [23], thereby hindering the rate of bone turnover. The increase in the rate of bone turnover as well as the unbalance between bone formation and resorption are known to have major implications in the bone tissue mechanical properties following the loss of ovarian hormones and are cornerstones of the pathophysiology of post menopausal osteoporosis [24]. Excess bone resorption leads not just to a progressive reduction in bone tissue volume, but also affects the average bone tissue age, reducing the time available for secondary mineralization to occur [25]. This explains the existence of a negative correlation between bone mineral content and biochemical markers of bone resorption in our study, and a lower bone mineral density in OVX animals with also had the highest bone resorption.

Both bone volume and tissue mineralization degree, are known to be major determinants of the bone biomechanical performance [26], and our results showed that Tb.Th, calcium content and phosphate content were significantly correlated with young´s modulus and described 31.4% of its variance. Despite significant, the contribution of these properties to bone stiffness was shown to be lower than what was previously reported by others [26-28]. Using human bone samples within a wide range of compositions regarding to the predominance of cortical and trabecular bone tissue, Keller [27] showed that bone mineral composition, determined by ash weighting, was highly correlated (R^2>0.81) with bone stiffness. Years later, Hernandez et al [26] reanalyzed Keller´s data and included in their predictive model both ash weight and an estimate of BV/TV, and showed that both parameters together had an increased correlation with bone stiffness (R^2=0.97). More recently, Cory et al [28] also reported that mineral density and BV/TV, assayed by µCT, were able to describe about 75% of the rat bone mechanical properties. All these studies however used bone samples that were shaped prior to the testing procedure, and that therefore did not

represented whole bone specimens. Hence these results neglected the contribution of bone architecture and geometry to its biomechanical performance.

Our results in turn, showed that bone morphology contributed significantly to the femur ultimate strength with multiple regression analysis showing that FNW, FNAL, CFMA and bone length altogether described 47.1% of bone strength variance. Surprisingly, none of the remaining properties assayed were shown to be as well correlated with the femoral neck ultimate strength as bone geometry.

As bone grows throughout the rat lifespan, a higher bone length was expected in OVX and SHAM animals compared to BSL, which was in fact shown by our results. Nevertheless, femur length was also higher in OVX animals compared to SHAM, showing that loss of estrogen accelerated bone length increase. Interestingly, there is evidence that, together with lower BMD, there is a significant difference in several geometric properties of the femur between individuals with high and low fracture risk witch highlights the importance of geometry in determining bone strength. For instance, Gnudi and colleagues [29] compared post-menopausal woman with a history of hip fracture with healthy controls and reported that, in addition to the significantly lower BMD, women with previous hip fractures had a higher hip axis length, femoral neck width and neck shaft angle. As a result, the increase in FNAL was considered in this and other studies [30, 31] as a factor that contributes positively to bone fragility. One important result in Gnudi et al [29] study however was that all those geometric parameters were shown to be highly and inversely correlated with femoral neck BMD. In another study, using data derived from almost 15.000 men and women, Beck and colleagues [13] also showed very clearly the existence of an inverse relationship between cortical thickness and BMD at the femoral neck and periosteal width, with the femoral neck becoming consistently wider in each successive decade of age. Although these findings come from cross-sectional studies, data from a prospective study [32] also shows that while femoral neck BMD

decreased 1.9% yearly, cross-sectional moment of inertia increased by 3.1%, and that those with the highest bone loss were also those who showed the greatest periosteal expansion. What these studies suggest therefore is that rather than being a stationary bone feature, proximal femur geometry evolves alongside changes in bone mass and tissue mineralization degree.

In our study, OVX animals were shown to have a higher femur length and FNAL than age matched controls and the increase in these properties was show to be associated with increased femur strength. The longitudinal bone growth increase and the diaphyseal cross sectional area enlargement have been described previously following estrogen loss [33], and these adaptations have been suggested to compensate for the decreases in bone tissue volume and mineral content occurred after estrogen loss [34]. In Ahlborg et al [32] study, as a result of the inverse relationship between femoral neck BMD and cross-sectional area, despite significant decreases in BMD were detected as soon as 6 years after the menopause, bone strength did not decreased significantly until 14 years after menopause. Therefore, instead of being considered as factor that contributes positively to bone fragility, changes in proximal femur geometric properties might represent an adaptive response of the bone to maintain its mechanical strength. Indeed, direct biomechanical testing of human cadaveric femurs has shown that both FNAL and FNW are significantly and positively correlated with femoral strength [35], further suggesting that their increase is not responsible for higher bone fragility but conversely contributes to increased bone resistance to fracture.

Results from our study also showed that FNAL was negatively correlated with bone tissue calcium density ($r=-0.470$, $p<0.01$), proximal femur BV/TV ($r=-0.704$, $p<0.01$), Tb.N ($r=-0.578$, $p<0.01$) and whole femur RA ($r=-0.527$, $p<0.01$) and positively with Tb.Sp ($r=0.618$, $p<0.01$), evidencing the inverse relationship between bone geometry and bone mass and mineralization degree. In fact, despite the absence of differences in bone strength between groups, when

biomechanical performance was normalized to the bone volume fraction and to the degree of mineralization (Fig. 3) we saw that, in overall, OVX animals had a best biomechanical performance than BSL and SHAM. This result clearly suggests that in order to compensate for decreases in bone mineral density and loss of trabecular and cortical bone, the femur geometry adapted in order to maintain bone resistance. So, as bone tissue becomes scarce and under mineralized, mechanical stresses might be increased in the femoral neck which could stimulate periosteal bone apposition. Moreover, estrogen has been described to inhibit periosteal bone formation [36], which could also explain the increased periosteal apposition in OVX animals.

It is however noteworthy to emphasize that OVX and SHAM animal's femur properties were only analyzed 9 months following the surgical procedure. Therefore, although we have found no differences between OVX and SHAM animal's femoral neck strength, it is however possible that the compensatory geometrical changes that we have identified might only occur in a somewhat late stage following estrogen loss as in a previous study [37] no differences were found in femoral neck geometry between OVX and SHAM rats only 15 weeks following surgery. Moreover, in this study [37], it was shown that decreases in femoral neck strength were even more pronounced 5 weeks than 15 weeks following OVX, which might be a consequence of the lag time between increases in bone resorption and bone formation following estrogen loss [38].

In our study, RA measurements were not shown to be significantly correlated with the proximal femur biomechanical properties. We believe that the underlying cause for this lack of association was the simultaneous significant relationship between RA and bone mass, bone tissue mineralization and bone size. RA measurements were shown to be highly dependent on both bone tissue volume and mineralization degree (R^2=0.554). However, RA was also shown to be negatively correlated with FNAL witch was one of the significant determinants of bone

strength. Consequently, as bone FNAL and bone tissue properties contribute inversely to RA, it is likely that the net effect of the change of both features results in the absence of meaningful changes in RA.

The OVX rat has been widely used as a model for the study of estrogen induced bone loss in experimental studies [39]. Nevertheless, this animal model has some drawbacks which we consider important to mention, regarding the implications that they might have on our conclusions. As we referred previously, rats progressively grow throughout their lifespan, which however does not happen in humans. Therefore, it is possible that the mechanisms leading to the adaptive response in bone geometry that we observed in rats, might not be so meaningful in humans. Therefore, despite being a valid observation for rodents, our conclusion that bone geometry was altered in the absence of estrogen in order to compensate decreases in bone tissue properties, might not necessarily be transferable to humans, even thought there is evidence from previous studies suggesting that this kind of geometric adaptations also occur in adult humans [32], as well as in other species like the sheep [40], in order to compensate for deficits in bone tissue quality. It is also important to mention that despite its validity as a bone mass estimation procedure [15, 41] RA is not a standard diagnostic tool for the assessment of fracture risk, and has been mainly used for investigative purposes. Therefore, it should be noted that our results regarding the effectiveness of bone mass measurements based on 2D imaging techniques for discriminating proximal femur biomechanical properties, should be viewed considering this limitation, i.e. that we have not used the most broadly available 2D imaging technique, which is DXA.

In conclusion, our results showed that while bone stiffness was significantly associated with the proximal femur trabecular architecture and bone tissue degree of mineralization, whole bone strength was especially associated with bone size and femoral neck geometry. Importantly, RA measurements were not found to be significant predictors of the proximal

femur biomechanical performance. Our results also showed that with estrogen loss there was a significant increase in bone size and femoral neck length, which seems to have compensated the lower bone tissue volume and mineral content in OVX, thereby preventing a meaningful bone strength decline, which is a verification of the classical Frost´s mechanostat model [42] of bone adaptation to mechanical loading.

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Conflict of Interest

All authors declare to have no disclosures.

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Table 1. Mean and standard deviation of each parameter assayed.

	BSL	SHAM	OVX
Histomorphometry			
Ct.Th (µm)	477 ± 59.7	557 ± 51.3[a]	507 ± 47.7[b]
BV/TV (%)	42.1 ± 3.01	43.9 ± 3.61	30.1 ± 8.98[ab]
Tb.Th (µm)	94.4 ± 8.61	108.3 ± 12.87[a]	105.6 ± 12.41[a]
Tb.N (/mm)	4.5 ± 0.14	3.9 ± 0.22[a]	2.8 ± 0.59[ab]
Tb.Sp (µm)	130 ± 7.6	144 ± 8.6[a]	252 ± 81.5[ab]
Mineral content			
Calcium content (mg)	98.7 ± 6.92	106.7 ± 8.71[a]	107.3 ± 8.86[a]
Calcium density (%)	22.7 ± 0.87	23.7 ± 0.72[a]	22.0 ± 0.51[ab]
Phosphate content (mg)	36.9 ± 2.52	40.2 ± 1.99[a]	39.8 ± 3.64[a]
Phosphate density (%)	8.5 ± 0.28	8.7 ± 0.33[a]	8.2 ± 0.23[ab]
Calcium/ Phosphate ratio	2.7 ± 0.05	2.7 ± 0.06	2.7 ± 0.06
Biochemistry			
17β-estradiol (pg/mL)	88.9 ± 18.73	84.5 ± 17.40	18.4 ± 5.34[ab]
Osteocalcin (ng/mL)	89.9 ± 34.81	56.8 ± 20.23[a]	76.3 ± 29.20
CTX (ng/mL)	11.45 ± 1.931	10.86 ± 1.427	13.38 ± 1.760[ab]
Radiographic absorptiometry			
Femoral head (%)	147 ± 14.9	140 ± 10.4	131 ± 10.8[ab]
Femoral neck (upper área) (%)	98 ± 12.5	96 ± 9.6	104 ± 13.6
Femoral neck (central area) (%)	115 ± 15.7	108 ± 10.3	108 ± 11.9
Trochanter major (%)	131 ± 12.2	128 ± 10.8	122 ± 9.8
Trochanter minor (%)	141 ± 13.0	133 ± 8.7	129 ± 13.0[a]
Intertrochanteric region (%)	139 ± 13.7	132 ± 11.8	128 ± 9.4[a]
Total fémur (%)	122 ± 16.8	101 ± 5.9[a]	104 ± 11.9[a]
Geometry			
Length (mm)	34.2 ± 0.64	35.5 ± 0.34[a]	36.1 ± 0.28[ab]
FNAL (mm)	9.5 ± 0.54	9.9 ± 0.74	11.0 ± 0.70[ab]
Relative FNAL (%)	27.7 ± 1.32	28.0 ± 2.26	30.3 ± 1.92[ab]
NSA (^{o})	128.9 ± 3.08	129.1 ± 3.39	132.9 ± 3.80[ab]
FNW (mm)	2.6 ± 0.17	2.5 ± 0.14	2.5 ± 0.17
Relative FNW (%)	7.6 ± 0.39	7.1 ± 0.36[a]	6.9 ± 0.36[a]
CFMA (mm)	3.8 ± 1.09	3.9 ± 0.73	4.0 ± 1.22
Relative CFMA (%)	11.1 ± 3.18	10.4 ± 2.51	11.2 ± 3.19
Biomechanical testing			
Ultimate strength (N)	133.7 ± 26.17	123.3 ± 31.25	133.7 ± 23.45
Ultimate stress (MPa)	22.0 ± 3.52	24.5 ± 5.57	26.2 ± 3.78
Young´s modulus (MPa)	350.2 ± 162.88	504.8 ± 245.97	479.8 ± 163.74
Ultimate strain	8.5 ± 2.07	6.8 ± 1.52[a]	8.1 ± 1.83
Energy to fracture (MJ)	0.94 ± 0.24	0.78 ± 0.16	1.00 ± 0.46

a - $p<0.05$ versus BSL.
b - $p<0.05$ versus SHAM.

Table 2. Correlation coefficients between biomechanical properties and each parameter assayed

	Ultimate strength	Young´s modulus	Ultimate strain	Ultimate stress	Energy to fracture†
Histomorphometry					
Ct.Th (µm)	-0.189	0.318	-0.497**	0.154	-0.385*
BV/TV (%)	-0.195	0.078	-0.343	-0.242	-0.268
Tb.Th (µm)	-0.095	0.502**	-0.323	0.195	-0.313
Tb.N (/mm)	0.002	-0.326	-0.084	-0.339	0.019
Tb.Sp (µm)	0.034	0.176	0.192	0.235	0.071
Mineral content					
Calcium content (mg)	0.163	0.449**	-0.272	0.196	-0.356*
Calcium density (%)	-0.148	0.117	-0.521**	-0.019	-0.306
Phosphate content (mg)	0.168	0.389*	-0.177	0.180	-0.288
Phosphate density (%)	-0.128	0.029	-0.359*	-0.042	-0.196
Calcium/ Phosphate ratio	-0.050	0.185	-0.331	0.040	-0.271
Biochemistry					
Osteocalcin (ng/mL)	0.263	-0.312	0.406*	-0.061	0.335
CTX (ng/mL)	0.103	-0.318	0.511**	0.038	0.180
Radiographic absorptiometry					
Femoral head (%)	0.096	0.022	-0.169	-0.037	-0.124
Femoral neck (upper área) (%)	-0.078	0.046	0.121	-0.063	0.041
Femoral neck (central area) (%)	-0.077	-0.013	-0.002	-0.161	-0.021
Trochanter major (%)	0.051	0.053	-0.205	0.038	-0.030
Trochanter minor (%)	0.101	0.103	-0.258	-0.004	-0.156
Intertrochanteric region (%)	0.061	0.067	-0.145	-0.021	-0.097
Total fémur (%)	0.079	0.089	0.016	-0.148	-0.207
Geometry					
Length (mm)	0.470*	0.238	0.244	0.333	0.067
FNAL (mm)	0.559**	-0.076	0.344	0.187	0.297
Relative FNAL (%)	0.428*	-0.161	0.281	0.084	0.320
NSA (^{o})	0.226	0.082	0.177	0.163	0.121
FNW (mm)	0.504**	0.028	0.081	-0.062	-0.094
Relative FNW (%)	0.283	-0.076	-0.015	-0.196	-0.074
CFMA (mm)	0.258	-0.259	0.282	-0.044	0.194
Relative CFMA (%)	0.228	-0.282	0.259	-0.078	0.178

† Spearman's Rho correlation; *p<0.05; **p<0.01

Figure Captions

Figure 1. Diagram of the right femur x-ray image. a) Representation of the regions of interest (ROI) where radiographic absorptiometry was determined: total femur (dotted line), femoral head (1), upper area of the femoral neck (2), central area of the femoral neck (3), intertrochanteric region (4), trocanter major (5) trocanter minor (6) and standard referent (0). b) Representation of the femoral neck geometric parameters assayed: FNW – femoral neck width; CFMA – compression force moment arm; FNAL – femoral neck axis length; NSA – neck shaft angle.

Figure 2. a) Ovariectomized (OVX) and sham-operated (SHAM) animal's body weigth at the beginning and at the end of the 9 months of experimental procedure. b) Body weight variation in OVX and SHAM animals through the experimental procedure expressed in percentage change from initial body weight.

Figure 3. Comparison of the proximal femur biomechanical performance between BSL, OVX and SHAM groups after normalization for bone tissue calcium density (Ca^{2+} density) and trabecular bone volume fraction (BV/TV). Brackets indicate significant differences between groups ($p<0.05$).

Figure 1
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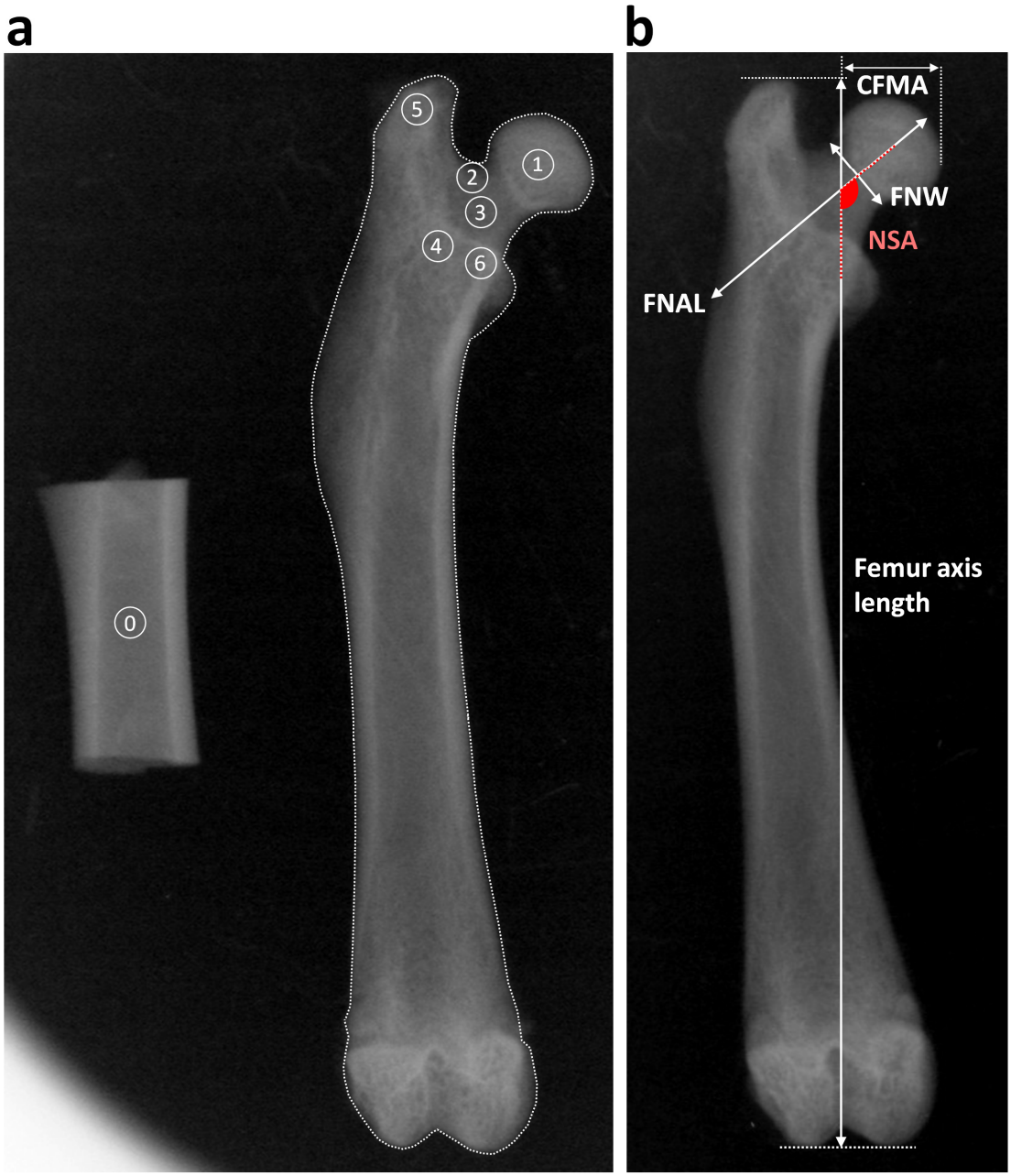

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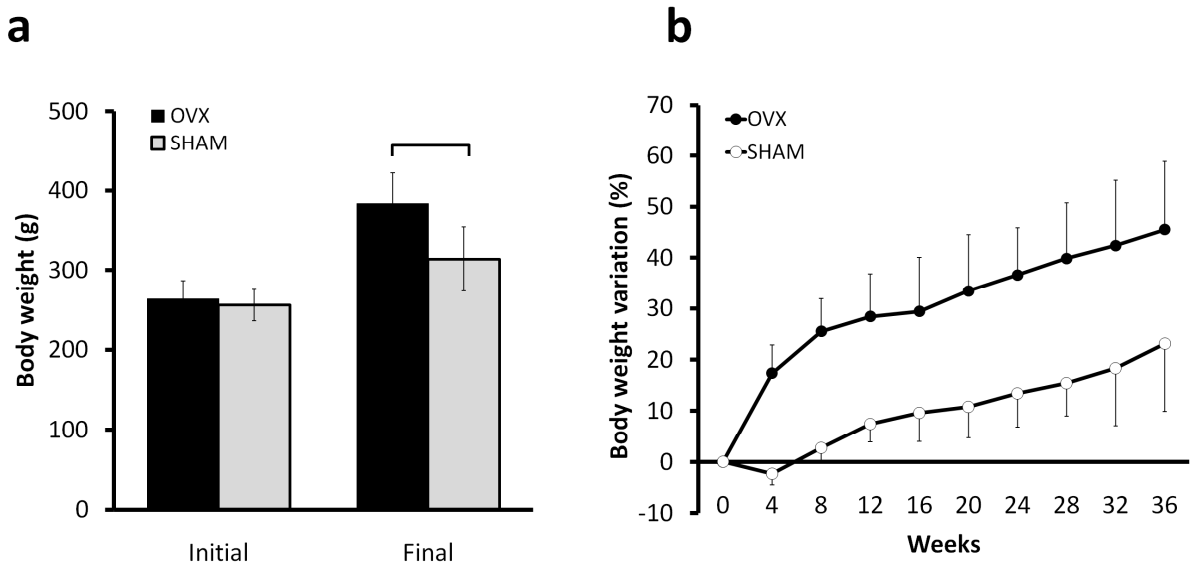

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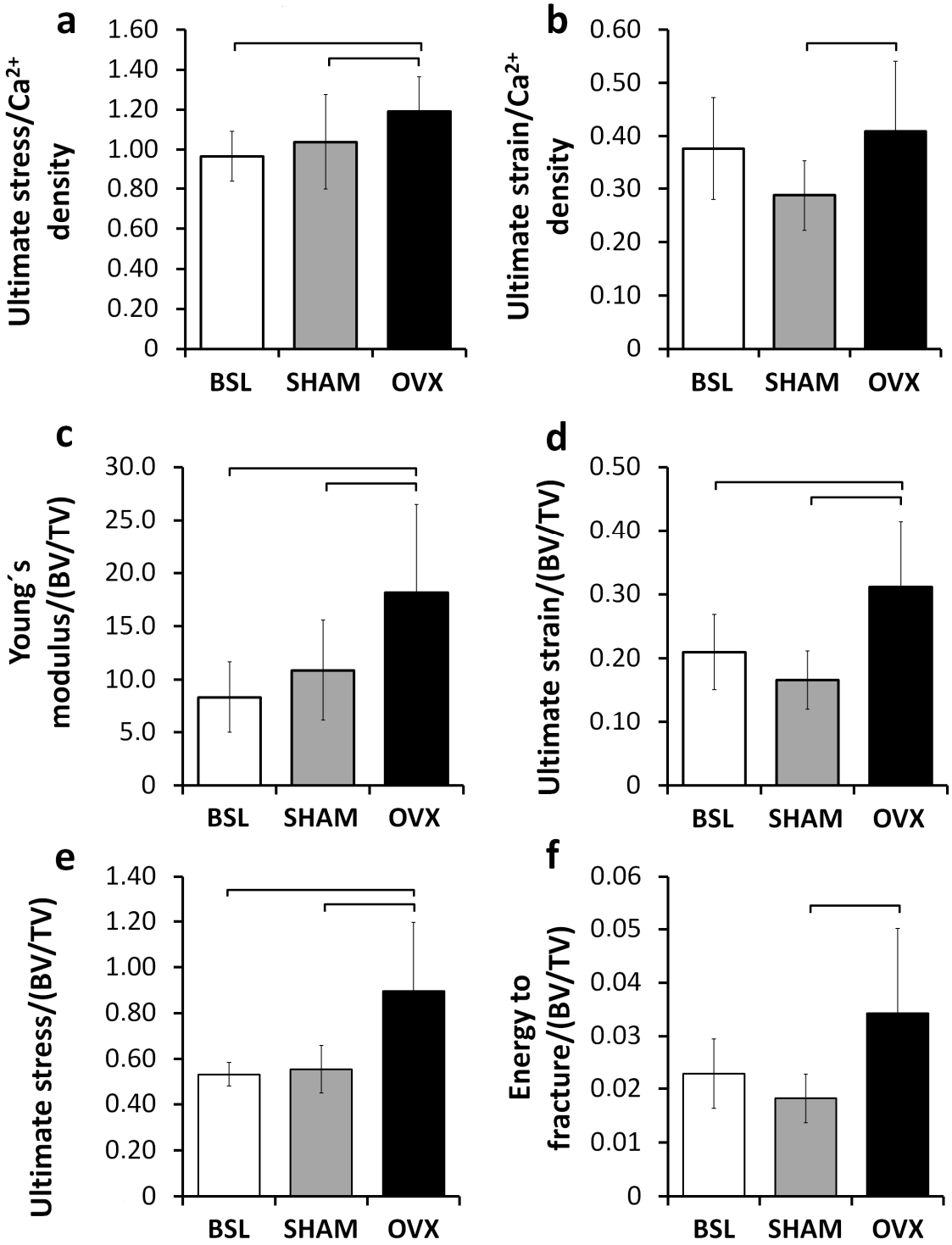

Study 4

Fonseca H, Moreira-Gonçalves D, Esteves JL, Ferreira R, Amado F, Mota MP, Duarte JA. **Voluntary physical activity improves proximal femur bone quality in adult ovariectomized Wistar rats**. (*Submitted to J Bone Miner Metab*)

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Abstract: The effectiveness of exercise to prevent bone quality losses following estrogen deficiency remains unappreciated by monitoring merely BMD. Our aim was to investigate how a broad set of bone properties are influenced by exercise in ovariectomized animals and to determine if exercise is able to prevent decreases in bone mechanical properties.
Thirty-two female Wistar rats (5-months old) were sacrificed as baseline controls (BSL, n=7), ovariectomized (OVX; n=13) or sham-operated (SHAM; n=12). OVX and SHAM animals were further exercised (OVX+EX, n=7; SHAM+EX, n=6) or kept sedentary (OVX+SED, n=6; SHAM+SED, n=6) for 9 months. At sacrifice, serum 17β- estradiol and bone turnover markers concentration was determined in the serum. Both femurs were collected for assessment of histomorphometry, femoral neck geometry, radiographic absorptiometry, bone mineral content and biomechanical properties. Exercise significantly prevented trabecular bone volume decreases in ovariectomized animals leading to the preservation of trabecular microarchitecture at both the femoral neck and distal femur regions. Bone mineral density was also shown to be higher, while bone resorption was lower in exercised than in sedentary ovariectomized animals. The preservation of trabecular microarchitecture and of bone mineral density contributed significantly to the higher Young modulus identified in ovariectomized animals that exercised regularly. The effects of exercise on bone tissue were substantially more pronounced in ovariectomized than in sham-operated animals. We conclude that voluntary exercise was able to prevent the long term bone mass losses in ovariectomized animals and to increase the femoral bone mechanical properties.

Opposed Reviewers:

Authors

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Title

Voluntary physical activity improves proximal femur bone quality in adult ovariectomized Wistar rats

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Abstract

The effectiveness of exercise to prevent bone quality losses following estrogen deficiency remains unappreciated by monitoring merely BMD. Our aim was to investigate how a broad set of bone properties are influenced by exercise in ovariectomized animals and to determine if exercise is able to prevent decreases in bone mechanical properties.

Thirty-two female Wistar rats (5-months old) were sacrificed as baseline controls (BSL, n=7), ovariectomized (OVX; n=13) or sham-operated (SHAM; n=12). OVX and SHAM animals were further exercised (OVX+EX, n=7; SHAM+EX, n=6) or kept sedentary (OVX+SED, n=6; SHAM+SED, n=6) for 9 months. At sacrifice, serum 17β- estradiol and bone turnover markers concentration was determined in the serum. Both femurs were collected for assessment of histomorphometry, femoral neck geometry, radiographic absorptiometry, bone mineral content and biomechanical properties. Exercise significantly prevented trabecular bone volume decreases in ovariectomized animals leading to the preservation of trabecular microarchitecture at both the femoral neck and distal femur regions. Bone mineral density was also shown to be higher, while bone resorption was lower in exercised than in sedentary ovariectomized animals. The preservation of trabecular microarchitecture and of bone mineral density contributed significantly to the higher Young modulus identified in ovariectomized animals that exercised regularly. The effects of exercise on bone tissue were substantially more pronounced in ovariectomized than in sham-operated animals. We conclude that voluntary exercise was able to prevent the long term bone mass losses in ovariectomized animals and to increase the femoral bone mechanical properties.

Keywords

Biomechanical testing

Femoral neck geometry

Histomorphometry

Exercise

Post-menopausal osteoporosis

Introduction

Osteoporosis is a prevalent metabolic bone disease in the elderly and postmenopausal women. Its major complication is fragility fracture occurrence due to bone mass loss and bone tissue properties deterioration.

Several pharmacological agents are available for the treatment of osteoporosis and their effectiveness is well demonstrated [1]. Nevertheless, as any drug, they also have adverse outcomes ranging from gastrointestinal discomfort to increased atypical femoral fracture occurrence [2]. Moreover, the cost of pharmacological treatments is a matter of concern as health care resources are inevitable limited. Therefore the struggle against osteoporosis should rely as much as possible on primary prevention based on non-pharmacological interventions such as exercise [3]. Several studies however show that exercise-induced bone gains occur especially during growth [4]. Nevertheless, there is also evidence that enhanced bone accrual during growth does not necessarily grant protection at old age [5]. Consequently, to be an effective preventive strategy, exercise should be performed throughout life.

However, several observational studies and clinical trials suggest that exercise has no effect or produces only minor BMD increments in the adult [6]. As the most widely available diagnosing technique, DXA derived areal bone mineral density (aBMD) has been the primary outcome in the majority of the studies for determining exercise-induced improvements in fracture risk. However, regardless of its value in diagnosing osteoporosis, BMD increases correlate poorly with fracture risk reduction following treatment [7], which suggests that its usefulness as a surrogate for treatment efficacy may be limited [8]. This results from the fact that bone strength depends on several material and structural properties [9] and changes in these properties are not necessarily captured by a shift in BMD [10] as we see in cases were fracture risk is reduced without increases in BMD [11] and in cases where increases in BMD do not reduce fracture risk [12]. Consequently, assessing treatment effectiveness by monitoring just BMD changes is insufficient and likely misleading and would instead be best achieved by

monitoring a broad set of bone properties that might change with treatment reducing thereby fracture risk. Therefore one of the aims of the present study is to determine if exercise is able to prevent losses in bone mass and degree of mineralization and to reduce resorption rate as well as to determine if these alterations are captured by radiographic absorptiometry (RA) measurements.

In a previous study by our group [13], we determined how several bone properties were associated with femoral neck biomechanical performance following estrogen loss, and it was shown that while there was a decrease in bone material properties, whole bone strength was not compromised, as these deficits were compensated by adaptations in bone geometry. Moreover, there is evidence that exercise is able to increase bone strength by increasing bone size [14] even without improvements in bone tissue quality [15]. Therefore, and considering that bone strength depends on the interaction between several properties and that some features might adapt in order to compensate for deficits in others, our objective in the present study is also to investigate how regular exercise might influence these several bone properties after estrogen loss and how does that affects bone resistance, i.e. whether if exercise affects predominantly bone material properties or instead if it promotes mainly geometrical adaptations.

Materials and methods

Animal models and experimental design

Thirty-two nulliparous Wistar rats (Charls River Laboratories, Barcelona, Spain) were either ovariectomized (OVX; n=13), sham operated (SHAM; n=12) or sacrificed as baseline controls (BSL; n=7) at 5-months age. Bilateral ovariectomy was performed by ventral approach under anesthesia with 4% sevoflurane while in sham-surgery ovaries were exposed but not removed. One week after surgery OVX and SHAM groups were each separated in two sub-groups and housed in different conditions for a period of 9 months. In one of the sub-groups, animals were individually housed in cages with activity wheel and distance counter (floor area 800 cm^2; Tecniplast) allowing them to perform voluntary running activity (OVX+EX, n=7; SHAM+EX, n=6). In the second sub-group, animals were housed in identical conditions (OVX+SED, n=6; SHAM+SED, n=6) except for the running wheel in the cage. Running distance in the wheel and body weight of each rat was recorded weekly. Standard rat chow (A04, SAFE, Augy, France) and water were provided *ad libitum* to all animals throughout the experimental period. Animals were maintained in an inverted 12h light/dark cycle in a humidity/temperature controlled environment. All procedures involving animal care were approved by the local ethics committee (University of Porto).

Animal sacrifice and tissue harvesting

Following the 9-month experimental period, all rats were anesthetized with 4% sevoflurane and sacrificed by exsanguination. All animals were 14 months old at time of sacrifice. Serum was separated from the blood by centrifugation at 1500rpm for 30min at 4^oC and used for assaying estrogen and markers of bone turnover concentration. Both femurs were removed and cleared of surrounding soft tissue. Right femur was used for assaying femoral neck geometry, RA, biomechanical testing and quantification of calcium and phosphate content. Left femur was used for assaying trabecular microarchitecture. Both hind limb *soleus* muscles

were also collected and weighted in a precision balance (Kern 870, Balingen, Germany) for assessment of muscle mass.

Biochemistry

Biochemical parameters were assayed in the serum using commercially available ELISA kits and a spectrophotometer (iEMS, Labsystems, Washington, USA) according to the manufacturer recommendations. 17β-estradiol concentration was assayed with Estradiol DE2693 ELISA kit (Demeditec Diagnostics, Kiel-Wellsee, Germany) for the confirmation of the OVX animal model. Assay range was between 9.7 and 2000pg/mL. Inter and intra-assay coefficients of variation (CV) are lower than 9.4% and 6.8%, respectively. Bone formation was assayed by measuring osteocalcin (OC) concentration with Rat-MID™ ELISA kit (Immunodiagnostic Systems, Boldon, UK). Assay range was between 50 and 1500ng/mL. Inter and intra-assay CV are lower than 7.7% and 5.0%, respectively. Bone resorption was assayed by measuring C-terminal telopeptides of type I collagen (CTX) concentration with RatLaps™ ELISA kit (Immunodiagnostic Systems). Assay range was between 7.7 and 188ng/mL. Inter and intra-assay CV are lower than 14.8% and 9.2% respectively. Positive controls for OC and CTX were assayed together with samples and their concentration was found to be within the manufacturer's quality control concentration limits.

Radiographs

Digital radiographs were taken from the right femur in the craniocaudal direction using dental radiographic equipment (CCX Digital, Trophy, Croissy-Beaubourg, France). The right femur was positioned over the sensor plate (DenOptix Imaging Plates, Sterling Heights, USA) at a 20cm distance from the X-ray-source. Exposure time and intensity were 0.08sec at 70Kv and 8mA. To ensure equal positioning between samples, all bones were placed with the caudal surfaces of both condyles and of the trocanter minor in contact with the film. A standard

reference made from a cortical bone section with 10mm of length was included in each radiograph for calibration purposes.

Radiographic absorptiometry

Bone mass was assayed in the right femur by RA as described previously [16] using imageJ software (NIH, Bethesda, USA). Correlation between RA and DXA derived aBMD has shown to be as good as between different DXA scanners themselves [17]. Briefly, each x-ray image was composed of pixels within a grayscale value between 0 and 255, were 0 is black and 255 is white. Whiter pixels correspond to higher radiopacity. Bone mass was determined in each region as the mean gray value (MGV) of the pixels within that region. For total bone mass, the boundary of the femur was manually traced and the whole bone defined as the region of interest (ROI). For the other analyzed regions, including the standard reference, a circular selection with 1.5mm of diameter was defined as the ROI and positioned according to anatomical landmarks. The MGV registered for each region was the average of 4 repeated measures. In order to consider differences in background noise, the MGV of the background in the vicinity of the ROI was subtracted to the MGV of each region analyzed. Results were expressed as percentage of the MGV of the standard reference. The regions analyzed were total femur, femoral head, upper and central area of the femoral neck, trocanter major and minor and intertrochanteric region (Figure 1a). The CVs between repeated measures were 0.05%, 0.26%, 1.32%, 0.17%, 1.45%, 1.50% and 1.59%, respectively.

Femoral neck geometry

Femoral neck geometry was assayed on the digital radiographs of the right femur by measuring standard geometric parameters [18] using ImageJ software, as described previously [19] (Fig. 1b). Briefly, femoral-neck axis length (FNAL) was the distance from the lateral aspect of the trocanter major to the medial surface of the femoral head. Femoral neck-shaft angle

(NSA) was the angle between the femoral neck and shaft axes. Femoral-neck width (FNW) was measured at the narrowest region perpendicular to the femoral neck axis. Compression force moment arm (CFMA) was the distance between femur axis and the medial surface of the femoral head. Results were the average of 2 repeated measures. Relative lengths were determined and expressed as percentage of femur length in order to allow comparisons independently of femur length. CVs between repeated measures were found to be among 0.07% (femur length) and 2.8% (CFMA).

Histomorphometry

Left femur proximal and distal thirds were sectioned with a circular diamond saw continuously wet in could PBS (pH 7.2) and immersed in a solution containing 4% paraformaldehyde, 2.5% sucrose (Sigma, St. Louis, USA), 0.1% gluteraldehyde (TAAB, Aldermaston, UK) and 10% EDTA (Sigma) in PBS (pH 7.2) at 4^{o}C during 3 weeks for fixation and decalcification. Bone samples were then dehydrated through graded ethanol (Panreac, Barcelona, Spain) concentrations and mounted in paraffin (MERK, Darmstadt, Germany). Six-µm-thick sections were obtained from the head and neck region of the proximal epiphysis in the coronal plane as well as from the intercondylar fossa region of the distal epiphysis in the sagital plane (Fig. 2). Sections were stained with H&E and viewed under a light microscope coupled to a digital camera (Axio Imager A1, Carl Zeiss, Oberkochen, Germany). Digital images were analyzed with imageJ. Standard histomorphometric parameters [20] were determined as the average of five sections per specimen and included trabecular bone volume fraction (BV/TV, %), trabecular number (Tb.N; /mm^2), trabecular separation (Tb.Sp, µm), trabecular thickness (Tb.Th, µm) and cortical thickness (Ct.Th, µm). Trabecular architecture and Ct.Th were determined in the region between the femoral head growth plate and the lesser trocanter.

Biomechanical testing of the femoral neck

Biomechanical properties were assayed by compression testing of the right femur head according to recommend guidelines [21] using a servo-hydraulic testing machine (TIRATest 2705, Schalkau, Germany) as described previously [22]. Each femur midpoint was determined and sectioned with a circular diamond saw. The proximal half was then fixed on the testing apparatus and a vertical load applied on the femoral head parallel to the diaphysis. A stabilizing preload was first applied at a rate of $0.1mm.s^{-1}$ until 5N was reached. Above this point the load rate shifted to $0.5mm.s^{-1}$ until failure. Bones were kept moist in PBS throughout the testing procedure. The load-deformation plot and bone ultimate strength (maximal load registered, N) were obtained directly from the test and converted to stress-strain. Ultimate stress was the maximum stress the bone could sustain and ultimate strain was the strain at failure. Young´s modulus was the highest slope of the stress-strain curve. Energy to fracture was the area under the load-deformation curve. CV for compression testing was 7.6%.

Bone mineral content and degree of mineralization

Bone mineral content was determined by quantification of the calcium and phosphate extracted during the decalcification of the right femur, as described elsewhere [23, 24]. Briefly, after compression testing, the proximal and distal halves of the right femur were collectively fixed overnight in 70% ethanol (Panreac), lyophilized during 4 days (EZ550Q, FTS Systems, Warminster, USA) and their dry weight determined with an analytical balance (Kern 870). They were then decalcified in 5mL of 2% formic acid (Sigma) during 10 days. In each day a 2mL aliquot of the decalcification solution was collected, the remaining discarded and replaced by 5mL of fresh solution. Calcium content was then determined as the sum of the calcium in each daily aliquot by atomic absorption spectroscopy (Perkin Elmer AAnalyst 100, Massachusetts, USA) after addition of 0.5% lanthanum chloride (Sigma). Degree of mineralization was the ratio between calcium content and both halves dry weight. Calibration solutions (Sigma) were read

between sets of samples to minimize effects of equipment drift. Recovery degree was found to be on average 99.1% and CV between repeated samples to be 2.84%. Phosphate content was determined by spectrophotometry (iEMS, Labsystems) based on the formation of molybdate complex using KH_2PO_4 solutions as standard (Sigma). Total phosphate content and density were calculated as described above for calcium. Average CV between repeated samples was 3.55%.

Statistical analysis

The Kolmogorov-Smirnov and Levene´s test were used to investigate within-group normality and homogeneity of variance of each variable, respectively. Comparisons between groups were performed by two-way-ANOVA with Bonferroni or Games-Howell (calcium density; energy to fracture) *post hoc* test whenever homogeneity of variance was verified or not, respectively. Whenever the necessary assumptions for ANOVA were not verified, either square (proximal femur Tb.N), natural log (distal femur Tb.Sp; head and trocanter major RA) or reciprocal (proximal femur Tb.Sp) data transformation was employed to reestablish the necessary assumptions. Results are expressed as mean ± standard deviation (SD) and differences were considered significant when $p<0.05$. Data was analyzed with PASW 18.0 (IBM, Armonk, USA).

Results

Means and standard deviations are detailed in tables 1 and 2.

Estrogen (17β-estradiol) concentration was significantly reduced in OVX animals when compared to both young (BSL) and age-matched sham-operated (SHAM) controls thus confirming the effectiveness of the ovariectomy. In addition there were no differences regarding estrogen concentration between OVX+SED and OVX+EX or between SHAM+EX, SHAM+SED and BSL (Table 1).

At the beginning of the experiments, when animals were 5 months old, there were no significant differences between groups regarding body weight. However, at the end of the 9 months of experimental period, when animals were 14 months old, average body weight had increased significantly in both OVX and SHAM animals ($p \leq 0.003$). Nevertheless increases were higher in OVX animals compared to SHAM counterparts (45.5±13.39% *vs.* 23.2±13.30% respectively; $p<0.001$) and higher in OVX+EX than in OVX+SED animals ($p<0.05$).

Average distance traveled per week in the running wheel was almost 10-fold higher in SHAM+EX compared to OVX+EX animals (34.93±11.70Kms *vs.* 3.95±0.85Kms respectively; $p<0.01$). Despite this discrepancy in distance traveled, there were no significant differences in *soleus* muscle weight between OVX+EX and SHAM+EX ($p=0.5$) neither between OVX+SED and SHAM+SED ($p=1.0$). In addition, *soleus* muscle weight was significantly greater in OVX and SHAM exercised animals when compared to OVX and SHAM sedentary counterparts ($p<0.05$), therefore confirming that animals housed with access to the running wheel were physically active whilst those to which access to the running wheel was denied were sedentary.

Proximal femur BV/TV was significantly lower in OVX animals compared to baseline and age-marched sham-operated animals (Table 2). Nevertheless, BV/TV was 65% higher in exercised vs. sedentary OVX ($p<0.01$) and 17% higher in exercised vs. sedentary SHAM animals ($p<0.05$) (Figure 2). Both OVX and SHAM groups had a lower Tb.N when compared to BSL. Moreover, Tb.N was also lower in the OVX group compared to SHAM. Nevertheless in OVX

animals, Tb.N and Tb.Th were 35% and 23% higher while Tb.Sp was 41% lower in exercised compared to sedentary counterparts while in the SHAM group only Tb.Th was 20% higher in exercised compared to sedentary counterparts. Proximal femur Tb.Th in OVX+EX and SHAM+EX groups, but not in sedentary controls, was also 22% and 24% higher than in baseline controls, respectively. In OVX and SHAM exercised animals, but not in sedentary, proximal femur cortical bone thickness (Ct.Th) was also 11% and 25% higher compared to BSL, respectively.

Distal femur BV/TV and Tb.N were also lower in OVX animals when compared to BSL and SHAM groups but significantly higher in exercised animals compared to sedentary counterparts. In OVX animals, BV/TV was 2-fold and Tb.N 81% higher in exercised than in sedentary animals while in SHAM animals, BV/TV was 30% and Tb.N 17% higher in exercised than in sedentary animals ($p<0.05$). Additionally, in the OVX animals Tb.Sp was 47% lower in exercised compared to sedentary animals ($p<0.05$). There were no differences between groups regarding distal femur Ct.Th.

Direct quantification of bone mineral content by atomic mass spectroscopy showed that all groups, with the exception of OVX+SED, had significantly higher calcium contents compared to BSL. However, after normalizing bone calcium content to bone tissue dry weight, only SHAM+EX animals showed a higher calcium density than BSL. In addition, calcium and phosphate density were also shown to be significantly lower in OVX animals when compared to SHAM counterparts. Nevertheless, calcium and phosphate density were significantly higher in OVX+EX than in OVX+SED animals (3% and 4% respectively; $p<0.05$). Moreover, OVX+SED group was the only to evidence a significantly lower calcium and phosphate density compared to BSL (5% and 6% respectively; $p<0.05$). There were no differences in calcium content ($p=0.544$) and density ($p=0.202$) between SHAM+EX and SHAM+SED. Calcium to phosphate ratio was identical throughout all groups.

Bone resorption, evidenced by serum CTX concentration, was higher in OVX+SED animals compared to all other groups, while in OVX+EX animals there were no significant differences compared to BSL or SHAM+EX animals. Serum CTX concentration was 17% higher in OVX+SED than in OVX+EX ($p<0.05$) but similar between SHAM+SED and SHAM+EX ($p=0.902$). Despite their significantly higher bone resorption rate, OVX+SED animals bone formation rate, evidenced by serum osteocalcin concentration, was not significantly increased compared to BSL ($p=0.766$) or to OVX+EX ($p=0.090$).

With age all animals increased bone size, as evidenced by the significantly higher femur length in all groups compared to BSL. Femur length however was 1.7% higher in OVX+EX than in SHAM+EX ($p<0.05$) and 2.5% in OVX+SED than in SHAM+SED ($p<0.01$). Nevertheless, there were no differences in femur length between exercised and sedentary animals. Both absolute and relative (after normalization to femur length) FNAL were greater in OVX than in SHAM animals, but significantly lower (≈10%) in OVX+EX and SHAM+EX compared to their sedentary counterparts ($p<0.01$). Moreover, relative FNAL was significantly higher in OVX+SED ($p<0.01$) and SHAM+SED ($p<0.05$), but not in OVX+EX ($p=0.087$) or SHAM+EX ($p=0.231$), compared to BSL. Although there were no differences in NSA between exercised and sedentary animals, NSA was 4.3% and 5.0% higher in OVX+EX compared to BSL and to SHAM+EX groups ($p<0.01$), respectively. There were no differences between groups regarding CFMA and absolute FNW, however relative FNW was ≈10% lower in exercised animals compared to BSL ($p<0.01$).

Regarding RA measurements, no differences were identified between SHAM+EX and SHAM+SED (Figure 3). Conversely, RA differences in total femur and in the femoral neck region were detected between OVX+EX and OVX+SED animals. Moreover in OVX+SED, but not in OVX+EX animals, RA at all sites analyzed, with the exception of the upper region of the femoral neck, was significantly lower than in BSL. Interestingly, total femur RA of the BSL group was higher than in all other groups, with the exception of OVX+EX.

Compression testing of the femoral head revealed that Young modulus was significantly higher in OVX+EX compared to BSL (51% higher; $p<0.05$) and to OVX+SED (72% higher; $p<0.05$) as well as significantly higher in SHAM+EX compared to BSL (62%; $p<0.05$) and SHAM+SED (66%; $p<0.05$). There were however no significant differences in ultimate strength, ultimate stress or energy to fracture between SHAM and OVX groups or between exercised and sedentary animals.

Discussion

Our experiments provide three main findings. First, in OVX animals, regular exercise dramatically influenced the bone properties, with exercised animals having a significantly higher trabecular bone volume and bone tissue degree of mineralization than sedentary animals as well as a significantly lower resorption rate. Second, the differences between exercised and sedentary animals were substantially more pronounced in OVX than in SHAM animals, even though SHAM+EX animals exercised 10-fold more than OVX+EX. Finally, exercise improved proximal femur Young modulus in both OVX and SHAM animals when compared to both age-matched sedentary controls as well as to 5-months old baseline controls. A discussion of each of these findings follows.

Regular physical activity prevents bone losses in ovariectomized rats

Our findings show that as expected, ovariectomy resulted in a dramatic bone mass loss and trabecular microarchitecture deterioration. However, bone mass losses in OVX animals were prevented to a great extent by regular physical activity, with histomorphometric analysis of both the proximal and distal femur showing notorious differences between exercised and sedentary animals. In the proximal femur, BV/TV (-65%), Tb.N (-35%) and Tb.Th (-23%) were all significantly lower in sedentary than in exercised OVX animals leading to a significantly higher Tb.Sp (+41%) in sedentary than in exercised animals.

In the distal femur the effects of regular physical activity were even more dramatic with sedentary animals displaying half the BV/TV and an 81% lower Tb.N than in exercised animals. Our histomorphometric results show therefore that bone tissue, following estrogen loss, maintains its responsiveness to mechanical stimulation, and that regular physical activity, although not being totally able to avoid bone mass losses, contributes significantly to its preservation. Moreover, the higher BV/TV in exercised animals was mostly attained by the preservation of Tb.N, which was 35% and 81% higher in the proximal and distal femur,

respectively, while Tb.Th was only increased in the proximal femur by 23%. As it was suggested previously [25], disruption of the connections between trabecular elements produces a disproportionate loss of bone resistance, for which the increased thickness of the remaining trabeculae can only partly compensate. Therefore exercise protected the most biomechanically significant component of trabecular microarchitecture [25] which is Tb.N.

Our results also show that the effects of exercise in OVX animals were limited to trabecular bone, as there were no significant differences between OVX+SED and OVX+EX animals regarding cortical bone thickness in both the proximal and distal epiphysis. Interestingly, previous studies performed on younger female rats that were exercised in a treadmill caring additional weigh on a backpack [26] have described precisely opposite findings, showing that proximal femur trabecular bone in OVX animals was unaffected by exercise whilst there was an increase in cortical bone, suggesting therefore that trabecular and cortical bone adaptations to exercise are certainly dependent to a great extent on age and exercise characteristics.

The rapid loss of trabecular bone mass is a well established consequence of estrogen deficiency [27] and results from the increased bone turnover and of the unbalance between bone formation and resorption [28]. By measuring the serum concentration of biochemical markers for bone formation (OC) and resorption (CTX), we were able to determine the bone turnover rate and balance. Our results show that bone resorption in OVX+EX animals was similar to that of BSL and SHAM+EX animals. Conversely, in OVX+SED animals, resorption rate was significantly higher than in OVX+EX and was in addition increased by almost 40% in comparison to sham-operated sedentary controls. These results therefore show that regular exercise was able to prevent the increases in bone resorption that follow estrogen loss. In a previous study [29], Lin and colleagues also demonstrated, in ovariectomized rats submitted to 60 days of hind limb immobilization, that bone resorption was significantly lower in the tibia from the load bearing limb than in the tibia from the opposite immobilized limb. In addition of being in agreement with these findings, our results further demonstrate that the effects of

regular physical activity on bone resorption were long lasting as bone resorption was maintained close to baseline levels even 9 months after ovariectomy.

In addition of leading to a progressive bone tissue loss, increases in bone turnover rate and excessive bone resorption also have a negative effect on bone tissue mineral content by reducing the time available for secondary mineralization to take place [30]. Consequently, by reducing the rate of bone turnover in OVX animals, we also hypothesized that exercise could have affected bone mineral content following estrogen loss. Previous studies have shown conflicting results in this regard, with exercise effects on bone mineral content varying according to exercise intensity, training duration and skeletal site analyzed [22, 31]. In our study, bone tissue degree of mineralization (calcium and phosphate density) was lower in OVX+SED compared to OVX+EX animals. Additionally, bone tissue mineralization degree was lower in OVX+SED, but not in OVX+EX animals, when compared to BSL controls, showing therefore that exercise was able to preserve bone mineral content following ovariectomy. Interestingly, total calcium content was even higher in OVX+EX animals than in BSL controls, which could be, at least partially, caused by the greater femur length in ovariectomized animals.

Besides decreasing bone mass, loss of estrogen is also shown to promote skeletal muscle mass losses [32] which may increase fracture risk due to the higher risk of fall [33]. Determination of the *soleus* muscle weight showed that muscle mass was significantly higher in exercised than in sedentary ovariectomized animals suggesting therefore that sarcopenia, a risk factor for both decreased bone quality and falls [33], was prevented by regular physical activity in ovariectomized animals.

Notoriously, the effects of ovariectomy and of exercise on bone tissue volume and mineralization degree were also detected by RA measurements, with a greater total femur and femoral neck RA detected in OVX+EX than in OVX+SED. No differences were however identified in the remaining analyzed regions of the femur. This result suggests that RA was

sensitive enough to detect the most meaningful differences between exercised and sedentary OVX animals, but unable to detect the more subtle differences evidenced between exercised and sedentary sham-operated animals.

Regular physical activity has a more pronounced effect on bone tissue of ovariectomized than of intact animals

Previous studies [34] show that estrogen receptor (ER) expression is down-regulated by low estrogen levels on bone cells, leading thereby to a lower ER expression in OVX animals. Importantly, ERs are involved in the signaling pathways that mediate bone tissue adaptations to mechanical loading [34-37]. For instance, proliferation is higher in osteoblasts that overexpress ER and is blocked by ER antagonists [35]. Additionally, mechanical loading of the ulna of wildtype mice results in significantly higher cortical bone increases than in the ulna of ER null mice [37]. Recent studies also suggested that ERs are involved in Wnt/β-catenin signaling [36], regulating thereby bone tissue adaptations to mechanical loading [38]. Together these findings suggest that ovariectomy might lead to a decrease in bone tissue responsiveness to mechanical loading.

Our study however showed opposite results with substantially higher differences between OVX+EX and OVX+SED animals than between SHAM+EX and SHAM+SED animals. While in OVX animals, exercise affected proximal femur BV/TV (+65%), Tb.Sp (-41%), distal femur BV/TV (2-fold increase), Tb.Sp (-47%) and bone tissue mineralization degree (+3%), in SHAM animals the effects of exercise were only seen in proximal (+30%) and distal (+17%) femur BV/TV. Furthermore, differences in Young modulus between OVX+EX and OVX+SED were higher (72%) than between SHAM+EX and SHAM+SED (66%). These finding are quite interesting considering that SHAM+EX animals were significantly more active than OVX+EX, running per week a 10-fold higher distance (34.93±11.70Kms *vs.* 3.95±0.85Kms respectively; $p<0.01$). Therefore, OVX animals were not only more sensitive to the stimulus provided by exercise, as they were also

the ones that benefited the most with it, even though they performed only a fraction of the exercise performed by SHAM counterparts. As body weight in OVX+EX animals was higher than in SHAM+EX, it is possible that this additional weight bearing might have enhanced the effects of exercise on bone tissue of OVX animals.

Regular physical activity improves bone tissue quality in ovariectomized animals but does not promote significant geometrical adaptations

Biomechanical testing demonstrated that exercise was able to improve bone tissue quality in ovariectomized animals. Young modulus at the femoral neck was not only 72% higher in OVX+EX animals compared to OVX+SED, as it was also 52% higher when compared to baseline controls, showing therefore that exercise not just prevented the decrease, as it also improved bone mechanical properties in OVX animals.

In a previous study by our group [13], we have identified a significant correlation between the femoral neck Young modulus and Tb.Th ($r=0.502$, $p<0.01$), calcium content ($r=0.449$, $p<0.01$) and phosphate content ($r=0.389$, $p<0.05$) being these variables responsible for about 32% of the Young modulus variance. Our results now demonstrate that these several bone properties were all significantly increased in OVX+EX animals leading thereby to a significant improvement in femur biomechanical performance.

Our previous studies [13] have also shown that the femoral neck ultimate strength correlated significantly with femur length ($r=0.470$, $p<0.05$), FNAL ($r=0.559$, $p<0.01$) and FNW ($r=0.504$, $p<0.01$). A significant correlation between FNAL and FNW with femoral neck strength has also been identified by others [39]. Interestingly, FNAL was shown to be higher in sedentary than in exercised animals. This increase was observed in both SHAM and OVX animals, suggesting that favorable geometric adaptations occurred in sedentary instead of in exercised animals. Bone mechanical properties are the outcome of bone tissue properties as well as of the way bone tissue is spatially organized [9]. These is compelling evidence [39, 40],

suggesting that bone geometry changes synergistically with material properties, and that changes in bone geometry are often a compensatory strategy to preserve bone strength as a result of deficits in bone tissue amount and quality. The existence of an increased FNAL in both groups of sedentary animals therefore suggests that there was a change in the femoral neck geometry in order to compensate for decreases in bone material properties, thereby preventing decreases in bone ultimate strength in sedentary animals, which is a verification of the classical Frost´s mechanostat model [41] of bone adaptation to mechanical loading.

Summary and conclusions

Our experiments provide significant findings regarding the effects that voluntary exercise has in the prevention of bone tissue quality decreases as long as 9 months after ovariectomy. We demonstrate here that exercise was able to preserve femoral neck trabecular architecture, increased bone tissue mineralization degree and significantly decreased bone resorption rate in exercised OVX animals compared to sedentary counterparts. Skeletal adaptations to exercise were also shown to be more pronounced in OVX than in SHAM animals. Finally, exercise resulted essentially in improvements in bone tissue quality but not in proximal femur geometric adaptations, resulting in a significantly higher Young modulus, but not in significant increases in bone ultimate strength.

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Conflict of Interest

All authors declare to have no disclosures.

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List of Tables

Table 1. Group analysis for estrogen concentration, body weight and *soleus* muscle weight

	BSL	SHAM		OVX	
		SED	EX	SED	EX
17β-estradiol (pg/mL)	88.9 ± 18.73	82.3 ± 19.51	86.7 ± 16.57	20.4 ± 5.69 [ac]	16.7 ± 4.78 [ac]
Initial body weigth (g)	262.0 ± 16.51	269.8 ± 16.70	241.8 ± 9.61	267.1 ± 28.72	260.3 ± 18.32
Body weight at sacrifice (g)	----- -----	324.7 ± 39.67	303.2 ± 41.27	363.5 ± 31.79	400.9 ± 36.61[c]
Body weight variation (%)	----- -----	21.0 ± 11.19	25.3 ± 15.85	35.4 ± 7.13 [bc]	54.1 ± 11.27 [c]
Soleus muscle weigth (mg)	222 ± 23.0	211 ± 23.5[b]	288 ± 26.6[a]	210 ± 20.6 [b]	261 ± 31.3[a]

Results are mean ± standard deviation. a - p<0.05 *versus* BSL; b - p<0.05 *versus* EX counterpart; c - p<0.05 *versus* SHAM counterpart. BSL – baseline control (5-months old); OVX – ovariectomized; SHAM – sham-operated; EX – exercised; SED – sedentary.

Table 2. Descriptive statistics and group comparisons for bone properties assayed

Variable	BSL	SHAM		OVX	
		SED	EX	SED	EX
Proximal Femur Histomorphometry					
Ct.Th (µm)	477 ± 59.7	511 ± 22.7[b]	596 ± 30.5[a]	484 ± 50.0	527 ± 38.6[ac]
BV/TV (%)	42.1 ± 3.01	39.0 ± 5.06[b]	45.7 ± 3.48	22.3 ± 4.89[abc]	36.8 ± 5.38[ac]
Tb.Th (µm)	94.4 ± 8.61	97.8 ± 7.95[b]	117.0 ± 8.95[a]	94.1 ± 3.97[b]	115.4 ± 7.03[a]
Tb.N (/mm)	4.47 ± 0.14	3.97 ± 0.24[a]	3.91 ± 0.22[a]	2.36 ± 0.43[abc]	3.18 ± 0.41[ac]
Tb.Sp (µm)	130 ± 7.6	155 ± 23.2[a]	140 ± 13.7	342 ± 84.2[abc]	204 ± 44.2[ac]
Distal Femur Histomorphometry					
Ct.Th (µm)	428 ± 71.0	383 ± 46.6	394 ± 62.2	406 ± 42.6	418 ± 45.2
BV/TV (%)	30.1 ± 4.78	22.1 ± 8.12[ab]	28.8 ± 3.40	5.6 ± 1.37[abc]	11.4 ± 3.90[ac]
Tb.Th (µm)	50.8 ± 5.47	56.7 ± 6.49[b]	64.3 ± 2.64[a]	59.6 ± 6.97[a]	65.9 ± 8.00[a]
Tb.N (/mm)	5.90 ± 0.40	3.82 ± 1.02[ab]	4.49 ± 0.52[a]	0.94 ± 0.21[abc]	1.69 ± 0.43[ac]
Tb.Sp (µm)	119 ± 15	223 ± 91[a]	161 ± 28[a]	1056 ± 248[abc]	556 ± 152[ac]
Mineral content					
Calcium content (mg)	98.7 ± 6.92	107.5 ± 4.69[a]	109.7 ± 6.19[a]	99.2 ± 5.54[bc]	114.7 ± 3.86[a]
Calcium density (%)	22.7 ± 0.87	23.22 ± 0.81	24.09 ± 0.16[a]	21.63 ± 0.24[abc]	22.25 ± 0.24[c]
Phosphate content (mg)	36.9 ± 2.52	38.02 ± 3.66	40.74 ± 2.39[a]	37.47 ± 3.01[b]	41.82 ± 2.96[a]
Phosphate density (%)	8.48 ± 0.278	8.52 ± 0.311[b]	8.95 ± 0.167[a]	7.96 ± 0.032[abc]	8.28 ± 0.209[c]
Calcium/Phosphate ratio	2.7 ± 0.05	2.7 ± 0.07	2.7 ± 0.04	2.7 ± 0.04	2.7 ± 0.07
Bone turnover markers					
Osteocalcin (ng/mL)	89.9 ± 34.81	56.7 ± 21.34[a]	56.9 ± 21.58	95.0 ± 34.48[c]	63.9 ± 18.85
CTX (ng/mL)	11.5 ± 1.93	10.9 ± 1.41	10.8 ± 1.58	14.9 ± 2.33[abc]	12.3 ± 1.20
Geometry					
Length (mm)	34.2 ± 0.64	35.47 ± 0.37[a]	35.53 ± 0.350[a]	36.35 ± 0.662[ac]	36.12 ± 0.297[ac]
FNAL (mm)	9.5 ± 0.54	10.5 ± 0.84[ab]	9.5 ± 0.42	11.5 ± 0.44[abc]	10.5 ± 0.50[ac]
Relative FNAL (%)	27.7 ± 1.32	29.9 ± 2.35[ab]	26.7 ± 1.05	31.8 ± 1.19[abc]	29.1 ± 1.55[c]
NSA (°)	128.9 ± 3.08	130.2 ± 4.04	128.0 ± 2.44	131.5 ± 4.32	134.5 ± 2.67[ac]
FNW (mm)	2.59 ± 0.168	2.55 ± 0.118	2.43 ± 0.139	2.62 ± 0.197	2.47 ± 0.127
Relative FNW (%)	7.57 ± 0.391	7.26 ± 0.184	6.84 ± 0.389[a]	7.19 ± 0.528	6.85 ± 0.337[a]
CFMA (mm)	3.81 ± 1.090	4.74 ± 1.576	3.36 ± 0.993	4.58 ± 1.263	3.51 ± 0.983
Relative CFMA (%)	11.1 ± 3.18	13.5 ± 4.38	9.5 ± 2.56	12.6 ± 3.31	9.8 ± 2.61
Radiographic absorptiometry					
Femoral head (%)	147 ± 14.9	142 ± 9.6	139 ± 11.9	125 ± 10.8[ac]	136 ± 8.1
Femoral neck (upper área) (%)	98 ± 12.5	95 ± 5.1	97 ± 13.1	93 ± 9.5[b]	114 ± 6.6[ac]
Femoral neck (central area) (%)	115 ± 15.7	106 ± 7.7	110 ± 12.9	95 ± 3.7[ab]	117 ± 4.4
Trochanter major (%)	131 ± 12.2	127 ± 10.8	129 ± 11.8	118 ± 8.7[a]	126 ± 9.7
Trochanter minor (%)	141 ± 13.0	134 ± 10.6	133 ± 7.4	122 ± 11.0[a]	134 ± 12.7
Intertrochanteric region (%)	139 ± 13.7	139 ± 11.5	132 ± 13.3	123 ± 8.4[a]	133 ± 7.5
Total fémur (%)	122 ± 16.8	100 ± 3.3[a]	103 ± 7.8[a]	94 ± 3.8[ab]	112 ± 9.2[c]
Biomechanical testing					
Ultimate strength (N)	133.7 ± 26.17	131.3 ± 36.02	104.9 ± 7.95	142.3 ± 20.74	126.4 ± 24.62
Ultimate stress (MPa)	21.97 ± 3.522	22.59 ± 2.753	23.96 ± 4.984	26.53 ± 3.972	25.89 ± 3.893
Young´s modulus (MPa)	350 ± 162.9	341 ± 97.9[b]	566 ± 230.9[a]	310 ± 38.3[b]	531 ± 127.6[a]
Ultimate strain	8.5 ± 2.07	7.5 ± 1.66	6.0 ± 1.00[a]	9.6 ± 2.70	7.4 ± 1.49
Energy to fracture (KJ)	935 ± 235.7	879 ± 123.7	680 ± 137.4	1209 ± 566.5	971 ± 514.1

Results are mean ± standard deviation. a - p<0.05 *versus* BSL; b - p<0.05 *versus* EX counterpart; c - p<0.05 *versus* SHAM counterpart. BSL – baseline controls (5-months old); OVX – ovariectomized; SHAM – sham-operated; EX – exercised; SED – sedentary. BV/TV - trabecular bone volume fraction; Tb.N - trabecular number; Tb.Sp - trabecular separation; Tb. Th - trabecular thickness; Ct.Th - cortical thickness; FNW – femoral neck width; CFMA – compression force moment arm; FNAL – femoral neck axis length; NSA – neck shaft angle; CTX- C-terminal telopeptides of type I collagen; OC – osteocalcin.

List of Figure Captions

Figure 1. Diagram of the right femur x-ray image. a) Representation of the regions of interest (ROI) where radiographic absorptiometry was determined: total femur (dotted line), femoral head (1), upper area of the femoral neck (2), central area of the femoral neck (3), intertrochanteric region (4), trocanter major (5) trocanter minor (6) and standard referent (0). b) Representation of the femoral neck geometric parameters assayed: FNW – femoral neck width; CFMA – compression force moment arm; FNAL – femoral neck axis length; NSA – neck shaft angle.

Figure 2. Micrographs of decalcified bone sections stained with H&E evidencing trabecular microarchitecture in: a) coronal sections of the femoral neck region; b) sagital sections of the femoral distal metaphysis. It is possible to appreciate a higher trabecular bone volume fraction (BV/TV) in both the proximal and distal femur of exercised animals in comparison to their respective sedentary counterparts. Bar is 500μm. BSL – baseline control (5-months old); OVX – ovariectomized; SHAM – sham-operated; EX – exercised; SED – sedentary.

Figure 3. Representative X-ray images of the animal´s right femur showing overall differences in radiographic absorptiometry between groups. Differences in radiographic absorptiometry measurements between exercised and sedentary animals were only identified in ovariectomized animals, namely for total femur and femoral neck region, but not between exercised and sedentary sham-operated animals. It is possible to appreciate the presence of the standard reference included in each radiograph for calibration purposes. BSL – baseline control (5-months old); OVX – ovariectomized; SHAM – sham-operated; EX – exercised; SED – sedentary.

Figure 1
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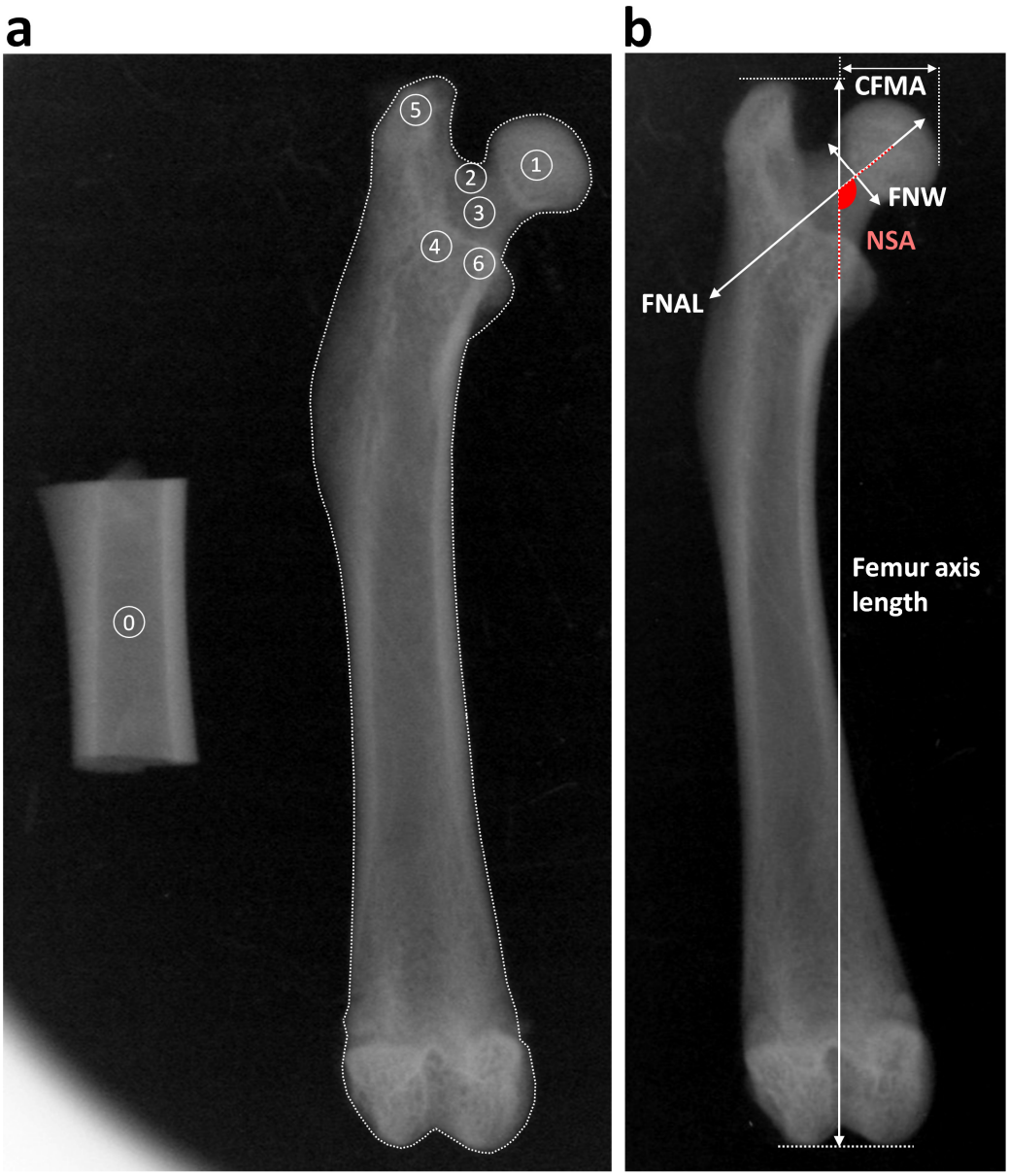

Figure 2
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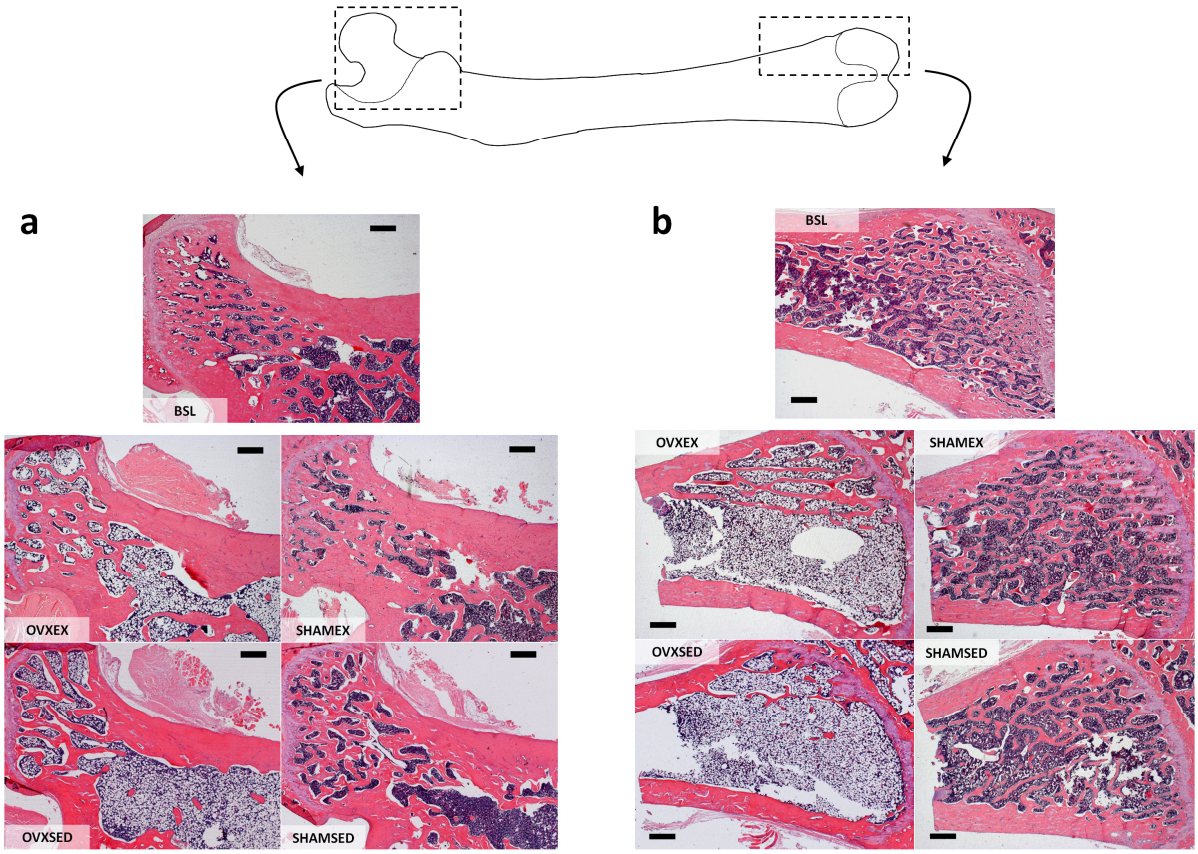

Figure 3
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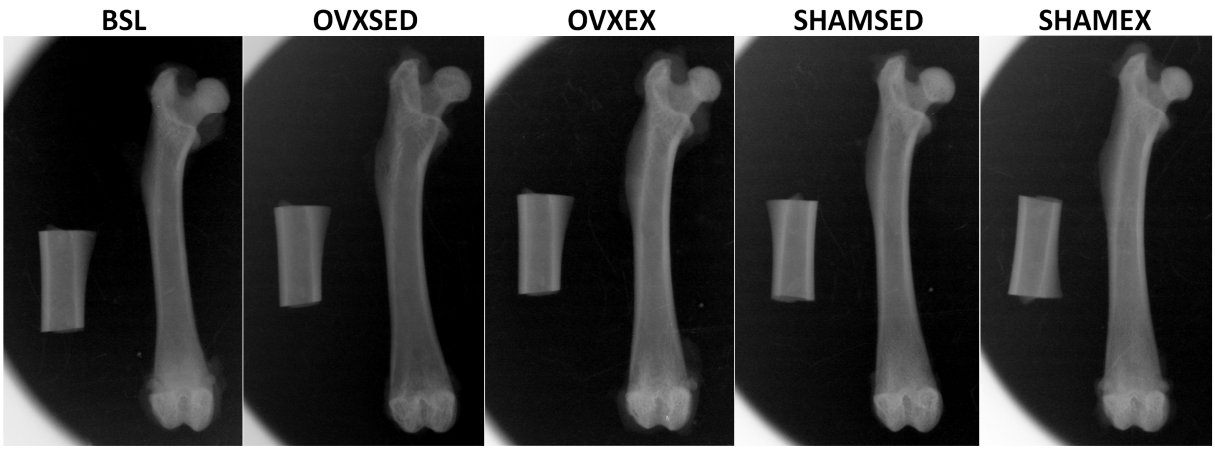

Study 5

Fonseca H, Moreira-Gonçalves D, Esteves JL, Viriato N, Vaz M, Mota MP, Duarte JA. (2011) **Voluntary Exercise has Long-Term In Vivo Protective Effects on Osteocyte Viability and Bone Strength Following Ovariectomy.** Calcif Tissue Int. 88(6):443-54

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ORIGINAL RESEARCH

Voluntary Exercise has Long-Term In Vivo Protective Effects on Osteocyte Viability and Bone Strength Following Ovariectomy

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Abstract Osteocytes are recognized as having a pivotal role in bone tissue homeostasis, and stimuli that increase osteocyte death result in decreased bone tissue quality. Previous in vitro studies have shown that mechanical stimulation prevents osteocyte death; however, in vivo evidence of this protective effect is limited. The aim of this study was to investigate if mechanical stimulation provided by voluntary exercise reduces osteocyte death caused by estrogen deficiency. Thirty-two female Wistar rats (5 months old) were either sacrificed as baseline controls (BSL, $n = 7$), ovariectomized or sham-operated and housed in cages with a voluntary running wheel (OVXEX, $n = 7$; SHAMEX, $n = 6$), or ovariectomized or sham-operated and housed in standard cages of equivalent size (OVXSED, $n = 6$; SHAMSED, $n = 6$) and sacrificed at age 14 months. Histomorphometric analysis of femur mid-diaphysis cortical bone revealed a significantly higher osteocyte number (N.Ot) and lower empty lacunae number (N.Lc) in both the OVXEX and SHAMEX groups compared to their SED counterparts. Intracortical porosity (Po.Ar) was also lower in both EX groups compared to their SED counterparts and significantly correlated with N.Lc ($r = 0.616$; $P < 0.001$). Three-point bending testing showed a significantly higher Young's modulus and ultimate stress in OVXEX compared to OVXSED and significant correlations between N.Lc and both yield stress ($r = -0.376$, $P < 0.05$) and ultimate stress ($r = -0.369$, $P < 0.05$) and between intracortical porosity and bone ultimate stress ($r = -0.451$, $P < 0.05$). Our results show that voluntary exercise prevented osteocyte death and that this protective effect was associated with increases in femur ultimate stress, which could be partially explained by decreases in Po.Ar.

Keywords Osteocyte death · Cortical porosity · Mechanical strain · Osteoporosis · Bone fragility

Since osteoblasts and osteoclasts are more actively involved in bone remodeling, they have been the most broadly studied bone cells. Substantial evidence, however, shows that osteocytes are also actively involved in both the local and systemic regulation of bone tissue metabolism. Osteocytes are considered the best candidates for detecting and coordinating local responses to mechanical and chemical signals due to their ubiquitous location within the bone matrix [1]. They contact through cytoplasmatic projections within canaliculi forming a syncytial network linked by adherent and gap junctions or hemichannels [2]. There is evidence showing that communication between osteocytes [3] and between osteocytes and osteoblasts or preosteoblastic cells regulates osteoblast differentiation, proliferation [4], and survival [2], controlling the rate of bone formation [5] by allowing the diffusion of second messengers, like nitric oxide [6], glutamate [7], and prostaglandins [8], throughout the osteocyte network. More recently, the identification of sclerostin secretion by osteocytes [9] and of

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its role as a regulator of bone formation [10] has reinforced the active role of osteocytes in bone remodeling.

Osteocyte viability appears to reduce with ageing [11], especially following estrogen deficiency [12], an effect that has been previously demonstrated in rats [13] as well as in humans [14]. Experimental animals show an increase in the number of apoptotic osteocytes as soon as 3 weeks after ovariectomy (OVX), which is reversed through estrogen-replacement therapy [13]. The mechanism through which estrogen protects osteocytes may include both classical receptor-dependent gene-activation effects [15] as well as receptor-independent effects due to the antioxidant properties of estrogen [16].

The decline in osteocyte number is paralleled by increases in bone fragility [17]. Experimental evidence demonstrates that osteocyte ablation rapidly leads to microfracture proliferation and decreased bone strength [18], showing that the presence of a viable osteocyte network is critical for maintenance of bone health. If in the short term an increase in bone fragility seems to be due to increased osteoclast recruitment by signals released by apoptotic osteocytes [19], in the long term an osteocyte decrease is linked with reduced bone remodeling and a consequent increase in microfracture proliferation [20] as well as reduced bone vascularity and hydration [21].

Despite detecting and converting mechanical into biochemical signals within bone, osteocytes seem to depend on these same mechanical signals for their own survival [22]. Tail-suspended mice show a rapid increase in osteocyte apoptosis, which is later followed closely by increases in osteoclast number and intracortical porosity and by reduced bone strength [23]. In vitro studies also demonstrate that cultured osteocytes subjected to cyclic mechanical stimulation have increased viability compared to unstimulated cultured osteocytes [22] and that fluid shear stress increases in osteocytes the Bcl-2/Bax ratio [24]. These findings indicate mechanical stimulation as a likely candidate for counteracting the extensive osteocyte death that occurs after estrogen loss. However, it is uncertain if these findings are also applicable to in vivo bones. Hence, further studies using in vivo models are needed to support the effectiveness of increased mechanical stimulation on osteocyte survival. These data would have meaningful clinical value in dealing with age-induced bone deterioration—namely, following menopause, where the lack of estrogen has a rapid deleterious effect on osteocyte viability [13], ultimately compromising bone strength [18]. If osteocyte death is a hallmark of bone fragility after menopause, osteocytes could be a target for prevention of bone fragility by the potential protective effects of mechanical stimulation.

Therefore, the objective of this study was to evaluate if in vivo mechanical stimulation induced by voluntary exercise reduces osteocyte death stimulated by estrogen deficiency in rats, with protective effects on bone mechanical properties. For this purpose, we used OVX female Wistar rats as a model in which osteocyte death is enhanced and voluntary daily physical activity performed in running wheels as a strategy to increase bone mechanical strain. Based on the results of previous in vitro studies [22, 24], we hypothesized that daily voluntary exercise would effectively counteract osteocyte death induced by estrogen loss and that this protective effect would prevent the deterioration of bone mechanical properties.

Materials and Methods

Animal Models and Experimental Design

All procedures involving animal care were approved by the local ethics committee and were in compliance with the "Guiding Principles in the Care and Use of Animals" approved by the Council of the American Physiological Society in 1991.

Following arrival, 32 nulliparous female Wistar rats aged 5 months (Charles River Laboratories, Barcelona, Spain) were randomly ovariectomized (OVX, $n = 13$), sham-operated (SHAM, $n = 12$) or killed as baseline controls following 1 week of quarantine (BSL, $n = 7$). Bilateral OVX was performed by the standard ventral approach [25] under anesthesia with 4% sevoflurane. In SHAM animals the ovaries were exposed but not removed. After 1 week of recovery, the OVX and SHAM groups were each divided into two subgroups of exercised (OVXEX, $n = 7$; SHAMEX, $n = 6$) or sedentary (OVXSED, $n = 6$; SHAMSED, $n = 6$). Rats in the exercise subgroups were housed in cages equipped with an activity wheel and distance counter (Tecniplast, Buguggiate, Italy), allowing them to run at will. The running distance of each rat was recorded weekly. Rats in the sedentary subgroups were housed in identical cages without a running wheel. All animals were housed individually and maintained in an inverted 12-h light/dark cycle in a humidity/temperature-controlled environment. Standard rat chow and water was provided ad libitum throughout the experimental period. The body weight of each animal was recorded weekly.

Animal Sacrifice and Tissue Harvesting

After 9 months of housing (at 14 months of age), OVX and SHAM rats were sacrificed by exsanguination under anesthesia with 4% sevoflurane, with blood collected from the vena cava used for assaying the estrogen concentration. Both femora were removed and cleared of surround soft tissue. The right femur was used for biomechanical testing

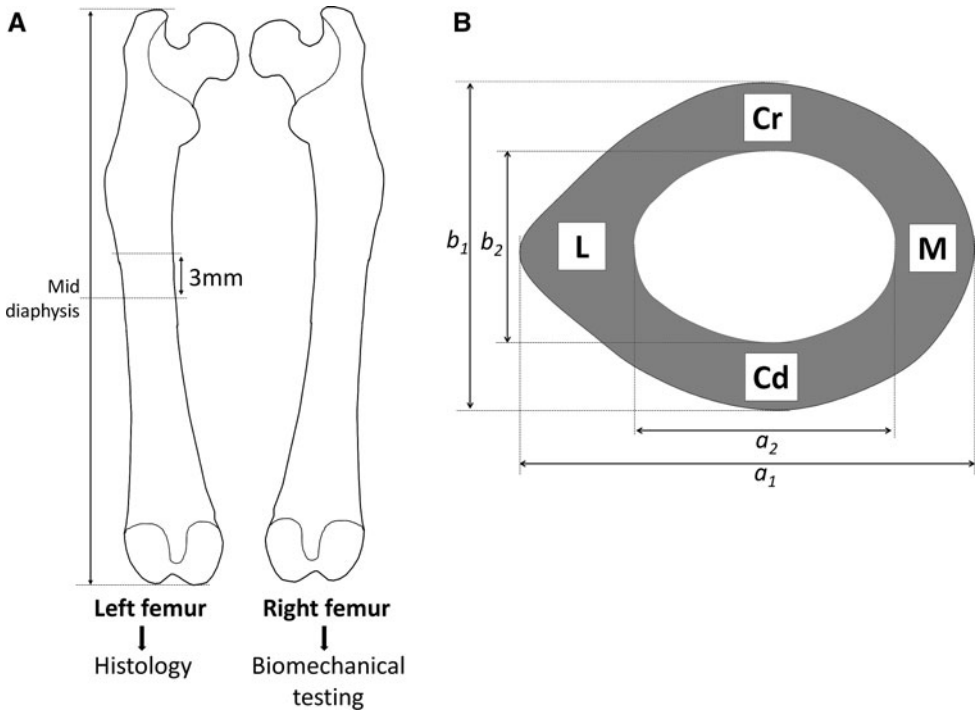

Fig. 1 **a** Femoral distribution for histology and biomechanical testing. **b** Representation of a femoral mid-diaphyseal transverse section. *White squares* represent osteocyte number (N.Ot) and empty lacuna number (N.Lc) assessment locations in each section. Recorded fields were 0.090 mm^2. *Arrows* show measurements used for femoral diaphysis cross-sectional moment of inertia ($I_{femoral}$) calculation. *Cr* cranial region, *Cd* caudal region, *M* medial region, *L* lateral region, a_1 lateromedial bone diameter, b_1 craniocaudal bone diameter, a_2 lateromedial marrow diameter, b_2 craniocaudal marrow diameter

and the left femur, for histology (Fig. 1a). Soleus muscles from both hind limbs were dissected and weighted collectively with an analytical balance (Kern 870; resolution 0.01 mg). The right soleus muscle was prepared for histology to evaluate the effectiveness of the exercise protocol by quantification of muscle fiber cross-sectional area.

Histology

The left femoral length was measured with a digital caliper (Powerfix, Leeds, UK, resolution 0.01 mm) from the proximal surface of the trochanter major to the distal surface of both femoral condyles. The midpoint of the diaphysis was determined, and a transverse section was cut with a circular diamond saw from the measured midpoint to 3 mm above this point (proximal direction) (Fig. 1a). The saw was continuously kept wet in cold PBS during the cutting procedure. Mid-diaphysis bone samples were then fixed and decalcified in a solution of 4% paraformaldehyde, 2.5% sucrose (Sigma, St. Louis, MO), 0.1% glutaraldehyde (TAAB, Aldermaston, UK) and 10% EDTA (Sigma) in PBS (pH 7.2) at 4°C for 3 weeks. They were further dehydrated in graded ethanol solutions and mounted in paraffin blocks. Bone samples were oriented to allow transverse cuts to be performed from the midpoint to the proximal direction. Each bone sample was serially sectioned in 6-µm-thick sections, each section collected at a distance of 30 µm away from the previous one. Five transverse sections were collected and stained with H&E.

The right soleus was fixed overnight at 4°C in a solution of 4% paraformaldehyde, 2.5% sucrose (Sigma), and 0.1% gluteraldehyde (TAAB) in PBS (pH 7.2), then dehydrated and mounted in paraffin blocks. Transverse sections (6 µm thick) were cut from the soleus mid-region and stained with H&E for fiber cross-sectional area determination.

Determination of Serum Estrogen Concentration

Serum was separated from blood samples by centrifugation at 4°C. Estradiol (17β-estradiol) concentration was assessed by solid-phase competitive binding ELISA using a commercially available kit (Estradiol ELISA DE2693, Demeditec Diagnostics, Kiel-Wellsee, Germany) and a spectrophotometer (iEMS and Labsystems, Washington, USA) according to the manufacturer's recommendations for confirmation of OVX animal model implementation. Briefly, 25 µl of serum and 200 µl of enzyme conjugate were dispensed in each well and incubated for 120 min at 25°C. After complete washing, 100 µl of substrate solution was added to each well and incubated for a further 15 min. Absorbance was read at 450 nm after addition of 50 µl stop solution. Duplicates were analyzed for each sample and triplicates for each standard. The assay range was 9.7–2,000 pg/ml.

Cortical Bone and Soleus Muscle Fiber Size Measurements

Cortical bone sections from the mid-diaphysis stained with H&E were examined under a light microscope coupled to a

digital camera (Axio Imager A1; Carl Zeiss, Oberkochen, Germany). Images were captured at 2.5× magnification and analyzed with ImageJ software (NIH, Bethesda, USA) for measurement of mid-diaphysis geometry: cortical width (Ct.Wi, μm), cortical bone tissue area (Ct.Ar, mm^2), marrow cavity diameter (Ma.Dm, mm), and bone diameter (B.Dm, mm). B.Dm and Ma.Dm on each section were determined as the average diameter from 30 direct measurements throughout that bone section.

Four images from the same section were captured with a 10× magnification objective for intracortical porosity area (Po.Ar) quantification as described previously [26]. Briefly, images were first converted to 16-bit gray, followed by segmentation into bone and pores using a fixed threshold. The margin of each pore was then traced and its area determined. Pores with an area less than 25 μm^2 (average size of osteocyte lacunae) were not considered. The pore area and cortical bone area of all images from the same section were summed to give total pore and bone areas, respectively, for that bone section. Results were then expressed as the summed area of all pores per cortical bone area (Po.Ar/Ct.Ar, %).

Osteocyte number (N.Ot) and empty lacunae number (N.Lc) were measured in four different regions of 0.090 mm^2 area in each bone section from images captured with a 40× magnification objective, as depicted in Fig. 1b. Results were expressed as numbers per area (N.Ot/mm^2, N.Lc/mm^2).

Transverse sections from the soleus muscle mid-portion were analyzed with a 40× objective. Cross-sectional area was determined in a total of 150 fibers per muscle. Results for each muscle were calculated as the average area of the 150 fibers analyzed.

Biomechanical Testing

Biomechanical properties of the right femur were assayed by three-point bending of the diaphysis according to recommended guidelines of procedure and calculation [27], using a servohydraulic testing machine (TIRATest 2705, TIRA, Schalkau, Germany). The supports in which the femora were placed were designed with AutoCAD software (Autodesk, San Rafael, CA) and made in aluminum and steel. Before each test, the femoral midpoint was determined by measuring with a digital caliper (Powerfix, Leeds, UK, resolution 0.01 mm) as described above. Femora were placed with the caudal surface on the lower supports. These were adjusted individually so that one was placed just distal to the trochanter minor and the other just proximal to the condyles. A bending load was applied to the femoral midpoint, perpendicular to its long axis in the craniocaudal direction by a steel crosshead coupled to a load cell. A stabilizing preload was first applied at a rate of 0.1 mm s^{-1} until 5 N was reached. Above this point the load rate shifted to 0.5 mm s^{-1} until bone failure. A computer-generated load–deformation curve was obtained for each test. Bones were kept moist in PBS throughout the testing.

Determination of Mechanical Properties

After direct acquisition of the extrinsic femoral properties, the load–deformation curve was converted to a stress–strain curve for determination of the intrinsic properties.

Femoral diaphysis cross-sectional moment of inertia ($I_{femoral}$, mm^4) was determined in left femoral histological sections by subtracting inner surface cross-sectional moment of inertia (I_{inner}, mm^4) from the outer surface cross-sectional moment of inertia (I_{outer}, mm^4) according to Eqs. 1 and 2 (a_1, lateromedial bone diameter; b_1, craniocaudal bone diameter; a_2, lateromedial marrow diameter; b_2, craniocaudal marrow diameter; all measurements in mm) (Fig. 1b):

$$I_{femoral} = I_{outer} - I_{inner} \quad (1)$$

$$I_{femoral} = \left[\pi/4 \times (a_1/2) \times (b_1/2)^3\right] - \left[\pi/4 \times (a_2/2) \times (b_2/2)^3\right] \quad (2)$$

Strain (ε) was calculated for each point of the load–deformation curve according to Eq. 3 ($b_1/2$, distance from the load application point to the bone neutral axis; Y, deformation; L, distance between the lower supports; all measurements in mm) (Fig. 1b):

$$\varepsilon = [12 \times (b_1/2) \times Y]/L^2 \quad (3)$$

Stress (σ) was calculated for each point of the load–deformation curve according to Eq. 4 (F, load applied to the specimen in newtons) (Fig. 1b):

$$\sigma = [F \times L \times (b_1/2)]/(4 \times I_{femoral}) \quad (4)$$

Intrinsic mechanical properties were then determined from the stress–strain curves generated. Ultimate stress (σ_U) was determined as the maximum stress the bone could sustain and is expressed in megapascals (MPa). Young's modulus was calculated as the slope of the stress–strain curve within the elastic region. Yield point was determined as the point of intersection between the stress–strain curve and a line with the same slope as Young's modulus and a positive offset of 0.03% strain. The coefficient of variation (CV) for three-point bending tests of the femur was determined as 4.21%.

Statistical Analysis

The Kolmogorov–Smirnov test was used to investigate within-group normality for a given variable. Levene's test was used to assess the homogeneity of variance. Statistical

analysis was performed by two-way analysis of variance (ANOVA) with Bonferroni post hoc test for pairwise comparisons between groups if normality or equality of variance existed. When normality or equality of variance assumptions were not met, root square transformation was employed (Ma.Dm, B.Dm, Ct.Wi). When transformation was inefficient to reestablish the necessary assumptions for ANOVA, the Kruskal-Wallis test was used for comparison (Po.Ar/Ct.Ar).

Correlation analysis was performed using Pearson's correlation coefficient except for Po.Ar/Ct.Ar, in which Spearman's rho was employed due to variable lack of normal distribution. Correlation between N.Ln and Po.Ar/Ct.Ar was determined using a quadratic equation model. Results are expressed as mean ± standard deviation (SD), and differences were considered significant at $P < 0.05$.

Results

Animals Weight Variation

At the beginning of the experimental procedure (5 months of age) there were no significant body weight differences between groups. Initial body weight was on average 269.8 ± 16.70, 241.8 ± 9.61, 267.1 ± 28.72, and 260.3 ± 18.32 g for the SHAMSED, SHAMEX, OVXSED, and OVXEX groups, respectively. As expected, during the experimental period (9 months of housing) all animals evidenced increases in body weight (Fig. 2d). Increases were thought more pronounced in OVX animals than in SHAM animals (45.5 ± 13.39 vs. 23.2 ± 13.30%, respectively; $P < 0.001$), in particular in the OVXEX group, in which mean body weight accrual was significantly higher than in both the SHAMSED and SHAMEX groups.

Soleus Muscle Weight and Fiber Size

OVXSED and SHAMSED animals displayed, at the time of sacrifice, a significantly smaller soleus muscle weight and mean fiber cross-sectional area than OVXEX and SHAMEX. Average soleus weight was 210.6 ± 23.54, 287.7 ± 26.63, 210.2 ± 20.65, and 261.0 ± 31.33 g for the SHAMSED, SHAMEX, OVXSED, and OVXEX groups, respectively (Fig. 2a). Average soleus fiber cross-sectional areas were 1206.5 ± 380.06, 2990 ± 734.28, 1081 ± 289.41, and 1950.2 ± 232.74 μm^2 for the SHAMSED, SHAMEX, OVXSED, and OVXEX groups, respectively (Fig. 2b). Fiber cross-sectional area was also higher in SHAMEX compared to OVXEX animals. This could be due to differences in the amount of running activity since fiber cross-sectional area and traveled distance were significantly correlated ($r = 0.765$, $P < 0.05$) and no significant differences in fiber cross-sectional area were identified between OVXSED and SHAMSED. Average running distance recorded per week was significantly higher (34.93 ± 11.70 km) in the SHAMEX group than in the OVXEX group (3.95 ± 0.85 km).

Serum 17β-Estradiol Concentration

Quantification of serum 17β-estradiol concentration confirmed the expected differences between OVX and both SHAM and BSL groups. Mean serum 17β-estradiol concentration in the OVXSED (20.4 ± 5.69 pg/ml) and OVXEX (16.7 ± 4.78 pg/ml) groups was significantly lower compared to the SHAMEX (86.7 ± 16.57 pg/ml), SHAMSED (82.4 ± 19.51 pg/ml), and BSL (88.9 ± 18.73 pg/ml) groups. There were no significant differences between the OVXSED and OVXEX or between the SHAMEX, SHAMSED, and BSL groups (Fig. 2c).

Cortical Bone Geometry

Analysis performed on femur mid-diaphysis cortical bone showed a significant increase in B.Dm with age only in OVX animals, regardless of their housing conditions since B.Dm of both OVXSED (3.42 ± 0.187 mm) and OVXEX (3.35 ± 0.082 mm) animals was significantly higher than that of BSL (3.12 ± 0.096 mm) animals (Fig. 3c). The same trend was verified regarding bone marrow cavity dimensions (Fig. 3a). Only OVXSED (2.27 ± 0.215 mm) and OVXEX (2.14 ± 0.148 mm) animals displayed significantly higher Ma.Dm compared to BSL (1.85 ± 0.140 mm).

In OVXSED animals the increase in endocortical dimensions (Ma.Dm) that took place with age surpassed the periosteal dimension (B.Dm) increase, leading to a significantly lower Ct.Wi compared to BSL (572.6 ± 34.19 vs. 639.10 ± 55.93 μm) (Fig. 3d). In OVXEX animals (607.3 ± 51.66 μm), however, there were no significant differences in Ct.Wi compared to BSL. Ct.Wi in OVXEX animals was only significantly decreased compared to SHAMEX (664.3 ± 24.85 μm). Despite the differences in B.Dm and Ct.Wi identified, Ct.Ar was similar in all groups. This indicates that the same amount of cortical bone was present in all groups, even though there were differences in its distribution around the bone axis (Fig. 3e). Ct.Ar was 4.98 ± 0.390, 5.23 ± 0.330, 5.11 ± 0.373, 5.33 ± 0.357, and 5.23 ± 0.422 mm^2 for the BSL, OVXEX, OVXSED, SHAMEX, and SHAMSED groups, respectively. Significant differences were not identified regarding Ma.Dm, B.Dm, Ct.Wi, and Ct.Ar between the EX and SED subgroups from both OVX and SHAM groups.

A significant increase in Po.Ar/Ct.Ar occurred with age in both OVXSED and SHAMSED animals but not in

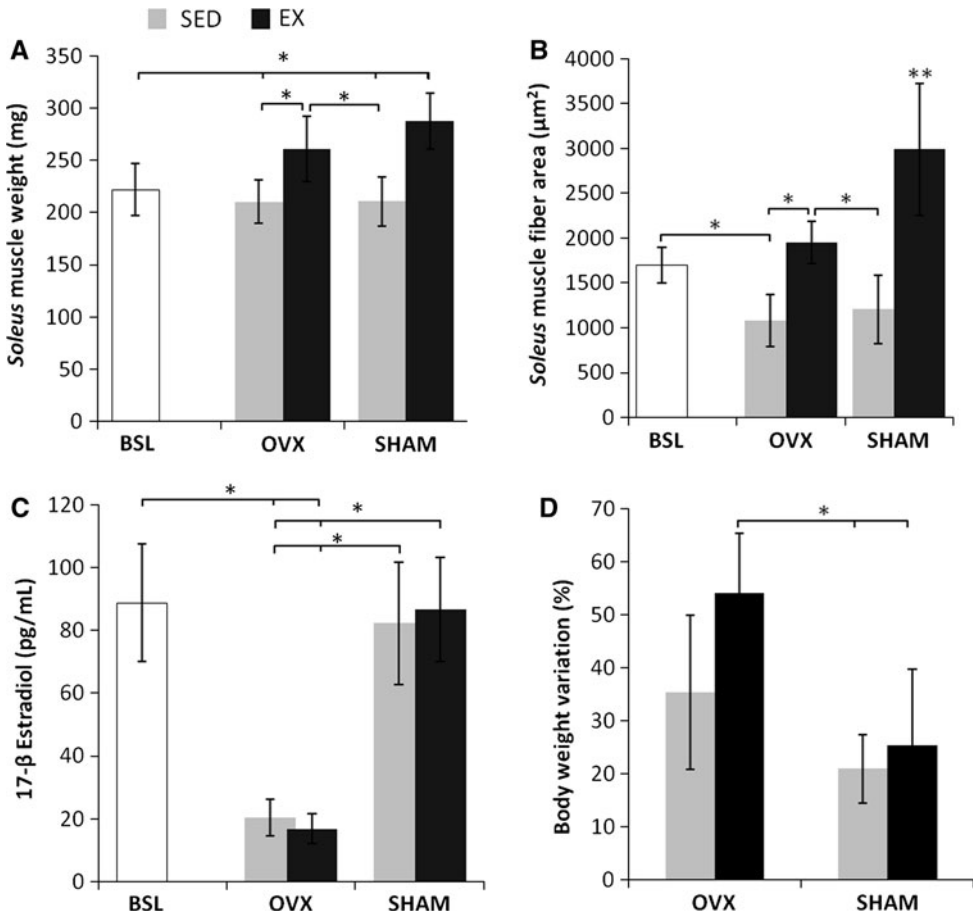

Fig. 2 Group description regarding soleus muscle weight and fiber cross-sectional area, serum 17β-estradiol concentration, and body weight variation. Both soleus muscle weight (**a**) and soleus muscle mean fiber cross-sectional area (**b**) were higher in EX animals compared to their SED counterparts, supporting the effectiveness of our voluntary physical activity model for inducing bone strain. The lower serum 17β-estradiol concentration in OVX animals (**c**) compared to both SHAM and BSL animals supports the effectiveness of the surgical procedure for inducing a state of low estrogen. *Differences between groups are statistically significant ($P < 0.05$), **statistically significant differences compared to all other groups ($P < 0.05$)

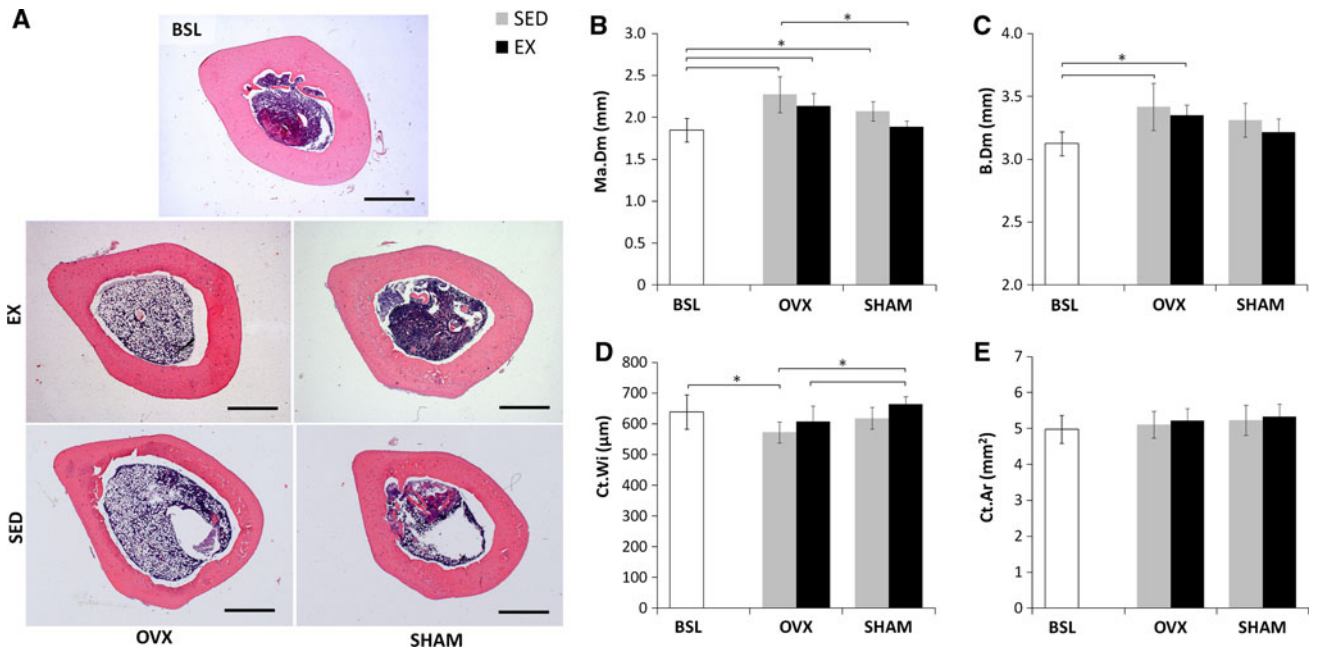

Fig. 3 **a** Photomicrographs showing femoral mid-diaphyseal transverse sections in each group. OVX animals displayed significantly higher Ma.Dm (**b**) and B.Dm (**c**) compared to BSL controls. **d** Despite these increases, when compared to BSL, Ct.Wi was significantly decreased only in OVXSED animals and not in OVXEX. **e** Regardless of the changes in cortical bone dimensions, there were no differences in the amount of cortical bone tissue (Ct.Ar) between groups. *Bars* = 1,000 μm. Sections were stained with H&E. *Differences between groups are statistically significant ($P < 0.05$)

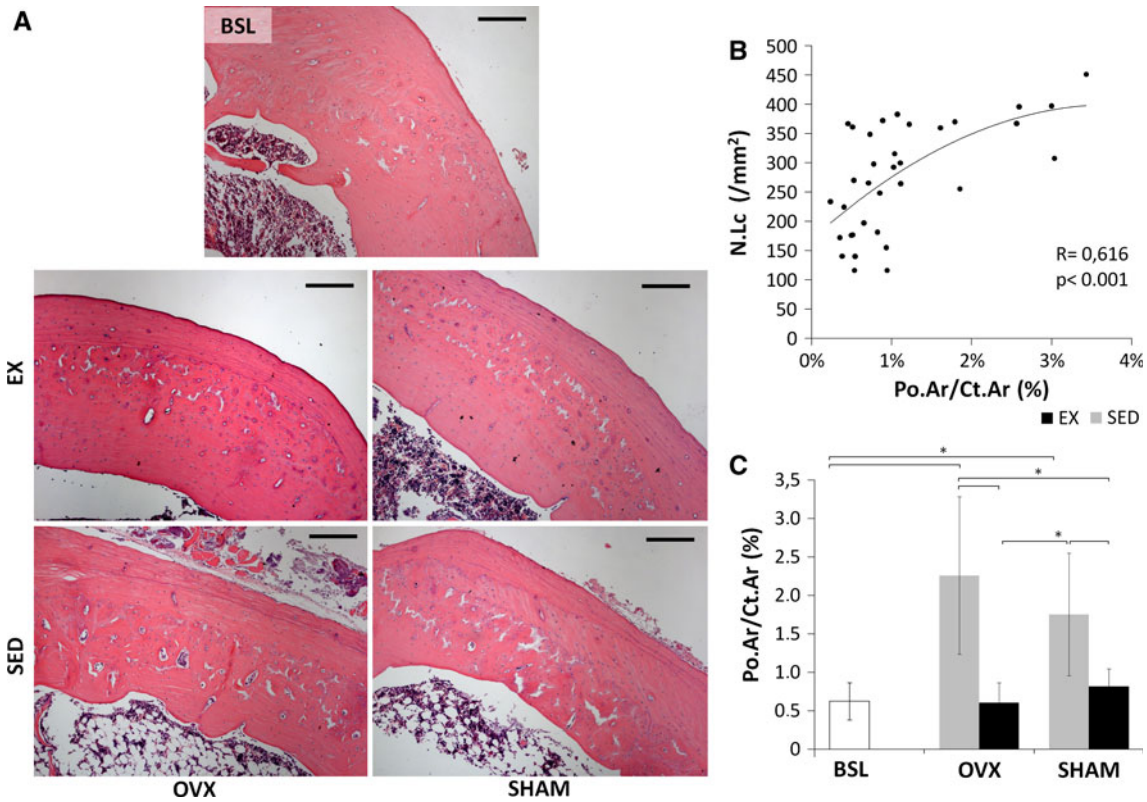

Fig. 4 **a** Photomicrographs showing a detail of femoral mid-diaphyseal axial sections. **b** Intracortical porosity was significantly correlated with empty lacuna number (N.Lc). **c** Intracortical porosity was significantly higher in SED animals compared to EX. In OVXSED there was an increase in bone marrow accumulation within cortical bone pores. *Bars* = 200 µm. *Differences between groups are statistically significant ($P < 0.05$)

OVXEX and SHAMEX animals (Fig. 4). Po.Ar/Ct.Ar in the OVXSED (2.3 ± 1.02%) and SHAMSED (1.8 ± 0.80%) groups was significantly increased compared to BSL (0.62 ± 0.24%). Moreover, Po.Ar/Ct.Ar was significantly smaller in OVXEX (0.61 ± 0.26%) compared to both OVXSED and SHAMSED groups and smaller in SHAMEX (0.81 ± 0.23%) compared to both OVXSED and SHAMSED groups. The increase in Po.Ar/Ct.Ar identified in SED animals, particularly in OVXSED (Fig. 4a), was accompanied by a noticeable increase in marrow accumulation within the intracortical compartment. Significant differences were absent between the OVXEX and SHAMEX and between the OVXSED and SHAMSED groups.

Osteocyte and Empty Lacunae Numbers in Cortical Bone

Housing conditions influenced significantly the number of osteocytes in femoral mid-diaphysis cortical bone, with SED animals displaying a significant reduction in N.Ot and an increase in N.Lc compared to their EX counterparts (Fig. 5). N.Ot was significantly higher in the SHAMEX group compared to SHAMSED (677 ± 59 vs. 619 ± 53/mm^2, respectively) and in the OVXEX group compared to OVXSED (632 ± 73 vs. 578 ± 71/mm^2, respectively). The OVXSED group was the only one to display a decrease in N.Ot with age since it was the only group to differ significantly from BSL (578 ± 71 vs. 632 ± 90/mm^2).

N.Lc was significantly smaller in BSL compared to all other groups, showing that a significant increase in N.Lc occurred with age. This increase was, however, significantly higher in both SED groups. N.Lc in the OVXEX (274 ± 56/mm^2) group was significantly smaller than in both the OVXSED (379 ± 63/mm^2) and SHAMSED (360 ± 59/mm^2) groups and in SHAMEX (275 ± 65/mm^2) it was significantly smaller than in both the OVXSED and SHAMSED groups. N.Lc was also significantly and positively correlated with Po.Ar/Ct.Ar ($r = 0.616$, $P < 0.001$) (Fig. 5b).

Biomechanical Properties

A significantly smaller Young's modulus and smaller ultimate stress were observed in the OVXSED group compared to OVXEX (Fig. 6). The OVXSED group showed also less ultimate stress and yield stress compared to BSL. Ultimate stress was also lower in OVXSED than in

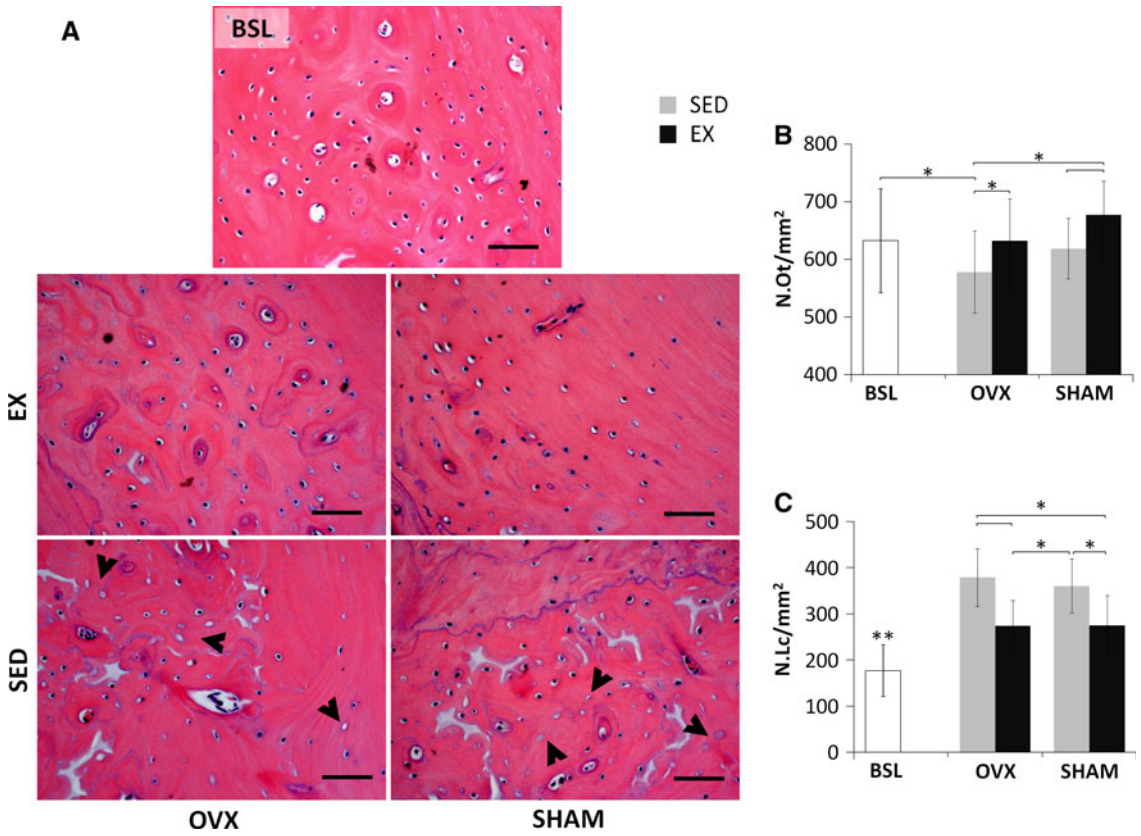

Fig. 5 **a** Photomicrographs showing a detail of femoral mid-diaphyseal axial sections evidencing differences in osteocyte (N.Ot) and empty lacunae (N.Lc) numbers. **b** Quantitative analyses of osteocyte number (N.Ot/mm^2). **c** Empty lacuna number (N.Lc/mm^2). *Arrowheads* indicate empty lacunae. Bars = 50 µm. *Differences between groups are statistically significant ($P < 0.05$), **statistically significant differences compared to all other groups

SHAMSED. SHAMEX was the only group to display a significant increase in Young's modulus compared to BSL. No significant differences were observed between the SHAMEX and SHAMSED groups for either biomechanical parameter evaluated despite a trend for higher values in the SHAMEX group.

Further analysis of correlation between the femoral biomechanical and geometrical properties showed that Young's modulus correlated negatively with Ct.Ar, B.Dm, and Ma.Dm (Table 1). Yield stress was also negatively correlated with both B.Dm and Ma.Dm and positively correlated with Ct.Wi. Ultimate stress correlated positively with Ct.Wi and negatively with B.Dm, Ma.Dm, and Po.Ar/Ct.Ar.

Regarding osteocyte and empty lacuna distribution in cortical bone, N.Ot was not correlated with any of the measured biomechanical properties. N.Lc in turn correlated negatively with both yield stress and ultimate stress.

Discussion

We present in this study evidence that voluntary exercise is able to provide a protective stimulus against osteocyte death caused by estrogen loss and that these cellular changes have beneficial effects on bone mechanical properties.

We used voluntary exercise as an approach to increase bone mechanical strain in vivo by housing the animals in different conditions throughout the experimental procedure. Mechanically stimulated animals were housed in cages with a running wheel, where they could voluntarily exercise, increasing bone mechanical loading while avoiding the drawbacks associated with forced exercise regimens on a treadmill [28]. The presence of muscle atrophy in animals housed in standard cages (OVXSED, SHAMSED) compared to their counterparts housed in cages with a running wheel (OVXEX, SHAMEX) confirms our model's effectiveness and shows that animals that had access to the running wheel were in fact more physically active. The greater muscle mass identified in EX animals suggests therefore that they experienced higher mechanical loading stimulation, which might have been due to both the higher ground reaction forces associated with the locomotor activity as well as to the higher muscle/tendon-derived forces [29].

The identification of differences in osteocyte numbers between animals housed in different conditions shows that the enhanced mechanical loading provided by exercise

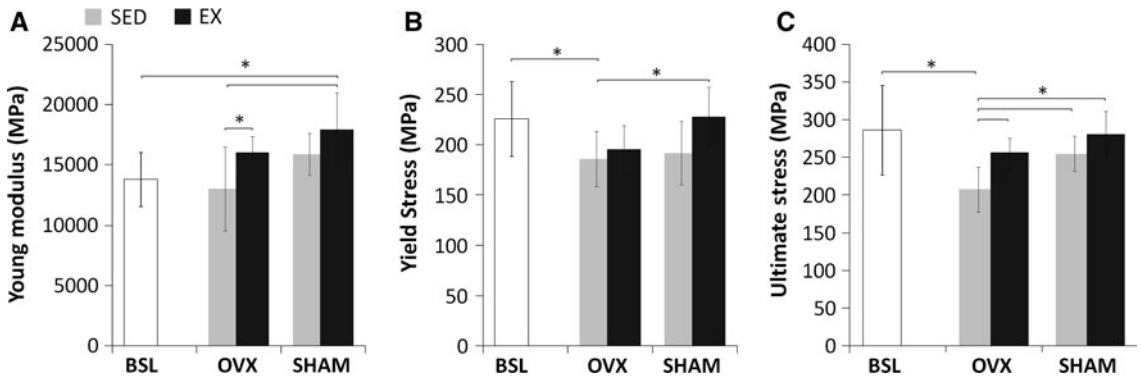

Fig. 6 Biomechanical properties of femoral mid-diaphysis assayed by three-point bending. Physical inactivity had mostly a detrimental influence on the femoral properties of OVX animals since no significant differences were identified between the SHAMSED and SHAMEX groups, although there was a trend for EX animals to have a higher Young′s modulus (**a**), ultimate stress (**b**), and yield stress point (**c**). *Differences between groups are statistically significant ($P < 0.05$)

Table 1 Correlation analysis between femur mid-diaphysis cortical bone properties and biomechanical outcomes

	Young's modulus		Yield stress		Ultimate stress	
	r	*P*	*r*	*P*	*r*	*P*
Ct.Ar	−0.352	0.048	−0.263	0.145	−0.253	0.162
B.Dm	−0.496	0.004	−0.807	<0.001	−0.779	<0.001
Ma.Dm	−0.387	0.029	−0.782	<0.001	−0.760	<0.001
Ct.Wi	0.082	0.654	0.472	0.006	0.468	0.007
Po.Ar/Ct.Ar[a]	−0.111	0.544	−0.337	0.059	−0.451	0.010
N.Ot	0.293	0.104	0.209	0.251	0.197	0.280
N.Lc	0.075	0.683	−0.376	0.034	−0.369	0.038

Ct.Ar cortical bone area, *B.Dm* bone diameter, *Ma.Dm* marrow diameter, *Ct.Wi* cortical width, *Po.Ar*/*Ct.Ar* intracortical porosity area per cortical bone area, *N.Ot* osteocyte number, *N.Lc* empty lacunae number

[a] Correlation coefficient was determined by Spearman's rho due to variable lack of normality. The remaining correlation coefficients were determined using Pearson's correlation coefficient

decreased osteocyte loss within the cortical bone. This effect was observed considering the significantly higher number of osteocytes present in EX animals compared to SED animals, but especially due to the smaller number of empty lacunae. Nevertheless, it is also possible that the higher number of osteocytes identified in EX animals might have resulted not only from an increase in osteocyte viability but also from an increase in the pool of osteoblasts that were available to become entrapped within the bone matrix and to differentiate as osteocytes as mechanical stimulation is known to increase the rate of mesenchymal stem cell replication and to bias their differentiation toward the osteoblastic lineage [30] as well as to decrease osteoblast apoptosis [31].

Osteocytes are long-lived cells that stay embedded within the bone matrix until they are eliminated by osteoclasts together with the surrounding bone matrix during bone resorption [32] or until they die from autolysis or apoptosis [33]. After osteocyte death, the lacuna in which the osteocyte cell body was housed remains empty, leaving a histological footprint that allows us to track osteocyte death [34]. Our results showed a significantly smaller number of empty lacunae in BSL animals in comparison to the remaining groups, indicating that with age there was progressive Ot death, which is in agreement with previous findings [35]. However, this increase was higher in SED animals compared to EX animals, showing that mechanical stimulation prevented osteocyte death.

Previous studies have demonstrated that increases in osteocyte death are associated with decreases in bone strength [23]. Our results are in agreement with these previous findings by showing that animals with fewer empty lacunae also had enhanced biomechanical properties. Furthermore, empty lacuna numbers correlated negatively with both yield stress and bone ultimate stress, demonstrating an inverse relationship between loss of osteocytes and bone resistance to fracture.

Osteocyte death may influence bone strength through different mechanisms [18, 19, 36, 37]. There is evidence that water within bone is to a great extent responsible for its viscoelastic properties, conferring much of the bone strength and resiliency by reducing stress during dynamic loading [11], with dehydrated bones showing a decrease in ultimate strength [27]. The major component of the pericellular matrix surrounding the osteocyte and its projections through the canalicular system is proteoglycan [38]. Considering proteoglycans high water affinity and osteocyte network extension, the canalicular system appears to be a major contributor to bone hydration. The death of osteocytes and the disruption of their associated pericellular matrix could therefore affect bone hydration status, compromising bone strength [21]. Finite element analysis

modeling shows that hydration produces a 2.5-fold increase in ultimate strength in cortical bone [39], and direct mechanical testing of cortical bone specimens with different degrees of hydration show a linear increase in stiffness with progressive water loss [40]. It is therefore possible that the correlation that we identified between empty lacuna number and both bone strength and yield stress could be related to a decrease in hydration status owing to osteocyte loss.

Our results also suggest that increases in osteocyte loss were associated with increases in intracortical bone resorption as empty lacuna number correlated positively with intracortical porosity, with higher numbers of empty lacunae being identified in animals that showed a higher intracortical porosity area. Osteocyte death, caused by estrogen withdrawal [15], tail suspension [23], or damage [19], is known to trigger osteoclast activation and to promote bone resorption within its vicinity, leading to increases in bone porosity. The significant correlation identified between intracortical porosity area and empty lacuna number could therefore result from the increase in intracortical bone resorption that follows osteocyte death.

The increase in intracortical porosity was also shown in our study to compromise bone strength as it was negatively correlated with bone ultimate stress. The significant negative correlation between cortical bone biomechanical properties and intracortical porosity has also been reported by others [41], and in a recent study [42] intracortical porosity was shown to be responsible for a 6% decrease in tibial stiffness.

However, despite the negative correlation of empty lacuna number and intracortical porosity with ultimate stress and the observation that both SHAMSED and OVXSED groups had an increase in empty lacuna number and cortical porosity, differences in bone biomechanical properties only reached statistical significance between OVXEX and OVXSED and not between SHAMSED and SHAMEX groups. As bone strength depends on the interaction between several bone properties [43], the significant differences only between OVXEX and OVXSED and not between SHAMEX and SHAMSED animals seems to be due to the effect that loss of estrogen has on other relevant properties for bone strength [44, 45]. Indeed, OVXSED animals showed a significantly smaller ultimate stress than SHAMSED animals, despite having similar cellular density patterns, which indicates a role of estrogen in the maintenance of other properties that determine bone strength and that were not considered in our study.

As biomechanical testing results reflect the intrinsic properties of the bones, differences in cortical bone geometry between groups do not explain the differences identified in bone strength. These differences were due to differences in the bone material properties. It was therefore interesting to note that all geometric bone properties measured were correlated with at least one of the biomechanical parameters measured. B.Dm and Ma.Dm in particular showed strong negative correlation coefficients with both yield stress and ultimate stress. It is known that a higher cortical perimeter has a dramatic influence on bone strength given that increasing a hollow cylinder's diameter provides exponential resistance increases to bending and torsion without necessary increases in bone mass [27]. It has been suggested, however, that increases in bone diameter associated with cases of ageing or menopause are the result of an adaptive mechanism that acts to maintain bone strength, compensating for decreases in bone mass or quality [46–48]. Therefore, and keeping in mind that our results for biomechanical testing reflect the intrinsic properties of bone, the strong negative correlation between bone diameter and bone strength suggests that a greater periosteal bone expansion was in fact a sign of bone tissue quality deterioration.

In summary, our results provide novel evidence that in vivo mechanical strain induced by regular voluntary exercise has a significant influence in decreasing long-term osteocyte loss in animal models. These long-term effects on osteocyte loss appear to be estrogen-independent as similar responses were seen in both OVX and SHAM animals. Furthermore, prevention of osteocyte death was associated with an increase in femoral bending ultimate stress, which could be partially explained by decreases in intracortical bone porosity. Voluntary exercise seems therefore to be a successful strategy for preventing, or at least delaying, osteocyte death and some of the typical features of skeletal involution associated with it, with benefits for bone strength.

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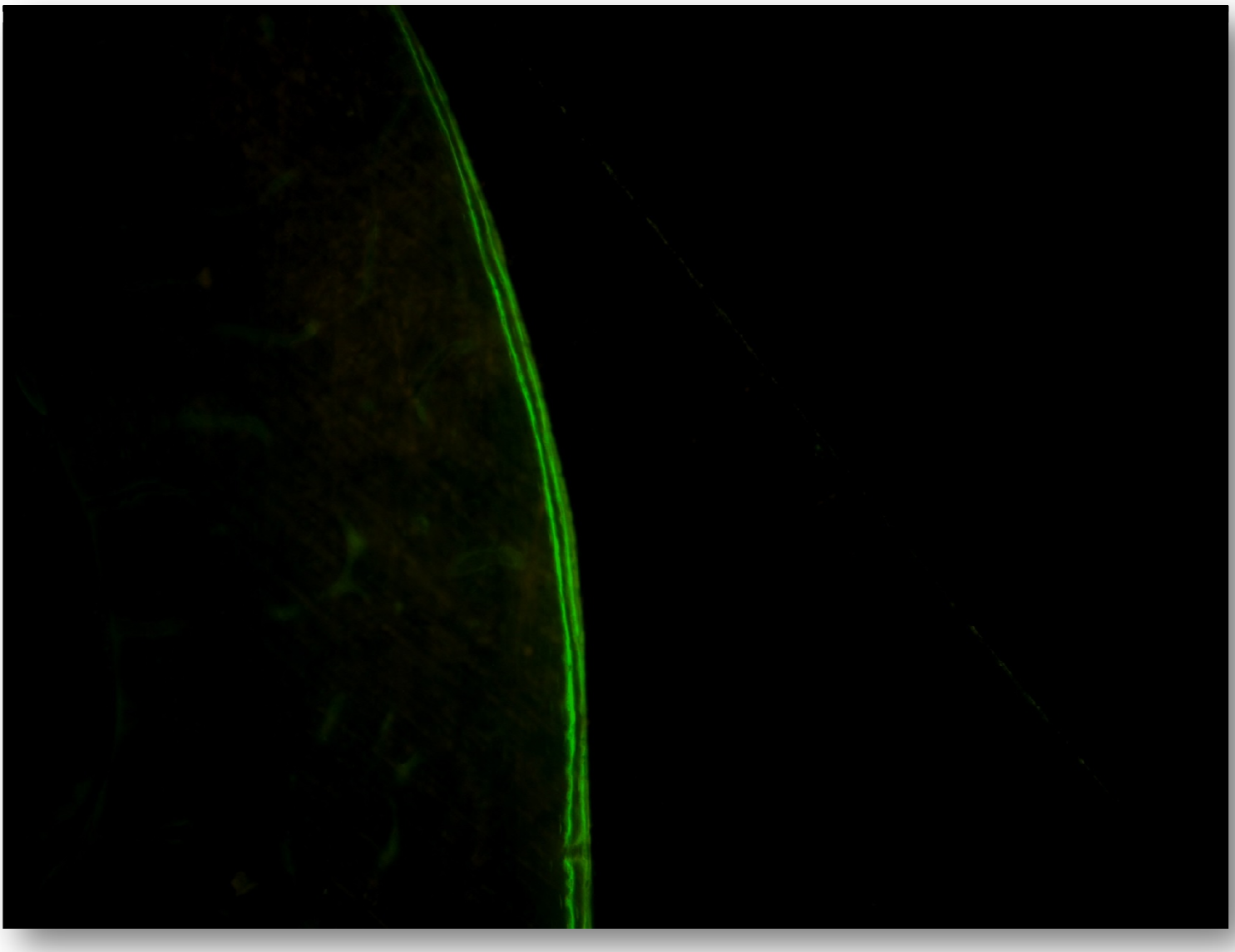
CHAPTER IV

General Discussion



1. General Discussion

All together the studies that we performed have provided a series of findings about the interplay that exists between age, low estrogen levels and sedentary behavior, and how each one affects the femur bone quality. Firstly, our results suggest that age as well as estrogen loss are associated with a significant decrease in voluntary physical activity levels. Secondly, results also suggest that some of the changes that occurred in the femur were not necessarily a direct consequence of the effects of age and/or low estrogen levels, since these changes were attenuated or even absent in animals that were allowed to grow old in physically active conditions. A general discussion of our findings addressing these main issues follows.

Voluntary physical activity decreases with age and estrogen loss

It is well established that estrogen exerts a pivotal role in the functionality and survival of bone cells [132, 493, 180, 508, 257, 526, 69, 226, 481, 229, 388, 629, 120, 193, 202, 554, 555], as well as on other organs that regulate calcium homeostasis [27, 400, 115, 568, 248, 141, 140], since the loss of estrogen leads to huge degenerative changes in bone structure in the elder and in post-menopausal women [456]. Nevertheless, other conditions are also known to affect significantly bone health, and among them, the lack of adequate amounts of mechanical stimulation is one the most well identified causes leading to bone loss [495, 304, 93, 426, 275, 145, 299, 577, 310, 59, 626]. Considering the importance of mechanical stimulation for bone health, we hypothesized that the increased bone fragility observed both with ageing and with estrogen loss might not just directly result from imbalances in calcium homeostasis or cell senescence *per se* but might also be a major outcome of the reduced mechanical stimulation associated with a decline in voluntary physical activity levels that usually accompanies age and estrogen loss.

The results from our first experimental study (study 1) demonstrated that estrogen levels are critical in the regulation of the animal´s spontaneous motor activity, with ovariectomized (OVX) animals running as much as 10-fold less than sham-operated (SHAM) counterparts. Despite that previous studies have also demonstrated that

motor activity is reduced as a consequence of low estrogen levels [254, 196, 131, 25, 529], our findings further demonstrate that this reduction was long-lasting, since the OVX animals running activity was consistently lower than that of SHAM animals throughout the time of study duration (36 weeks). In addition, and most notoriously, this reduction in physical activity levels was shown to be more pronounced than what was suggested previously by other studies [196, 254]. It was also interesting to notice that the presence of estrogen did not seemed to be a requirement to prevent decreases in skeletal muscle mass, being the reduced soleus muscle fiber cross-sectional area primarily associated with lower physical activity levels and not so much with the direct effects of low estrogen levels. Considering therefore the relevance of sarcopenia as a major risk factor for low bone mass [538, 586] and its association with balance problems and increased risk for fall [150], our findings are vital in the context of bone loss and fracture occurrence prevention since they reveal that the decreased skeletal muscle mass following estrogen loss may be, at least in part, prevented by counteracting the tendency for a reduction in physical activity levels.

Although not so dramatically as observed in females following estrogen loss, there is evidence suggesting that males also experience a decrease in voluntary physical activity levels with age [233]. In our second study we have addressed this issue by analyzing the pattern of voluntary physical activity of male mice from the age of 2 until 25 months old. Our results showed that during the first trimester of the experimental procedure the average daily distance traveled in the running wheel was of about 9 Km/day. Following this initial period of higher spontaneous physical activity, the average distance traveled in the wheel declined progressively throughout the succeeding 7 months, reaching thereafter an average daily distance of about 5 Km/day until the end of the experimental procedure. These results therefore suggest that voluntary physical activity levels in the male mice also tend to decrease as the animal grows old. However, it is noteworthy that even though the running distance declined with age, mice were still quite active at old age as they continued to perform a significant amount of running on the wheel. This result therefore suggests that experimental studies using animals housed in standard cages are at a great extent

imposing a sedentary lifestyle to the animal, which will certainly have consequences on the studied variables.

Having therefore gathered evidence supporting the concept that physical activity levels decrease with age and estrogen deficiency, the possibility that the degenerative changes in bone structure observed with age and low estrogen levels could also be an outcome of physical inactivity became stronger. Consequently, the next step was to characterize the changes in bone structure associated with age and estrogen loss, and to investigate to what extent were those changes influenced by an imposed sedentary behavior.

Age and estrogen loss affect several bone features that contribute significantly to bone resistance

To investigate the effects of age on bone structure, we analyzed the femoral bone from mice that were housed through life in physically active conditions (study 2). Male C57BL/6 mice were selected as animal models in this study because their age-related changes in skeletal mass and architecture have been suggested to resemble at a great extent those seen in human ageing [209]. As these animals were allowed to exercise at their will and were not forced to do it, these housing conditions mimicked, as much as possible, the pattern of physical activity that they would have in the wild habitat. On the other hand, animals that were housed in standard laboratory conditions and were therefore constrained to a sedentary behavior did not experienced only the effects of age *per se*, but also the influence of physical inactivity. Consequently, we assumed that the differences between young animals and animals that were allowed to exercise freely reflected the consequences of age and that the additional differences observed between aged active and aged sedentary animals would reflect the consequences of physical inactivity.

By comparing the femur structure between young (Y) and aged active (AA) animals we verified that with ageing, as expected, there was a significant deterioration of the trabecular microarchitecture, with AA animals displaying at both the proximal and distal epiphysis a significantly lower trabecular bone volume fraction (BV/TV),

trabecular number (Tb.N), trabecular thickness (Tb.Th) as well as a significantly higher trabecular separation (Tb.Sp). Despite these differences in trabecular microarchitecture, the analysis performed on the animal's pattern of cellular density revealed no differences in osteocyte density between Y and AA animals, suggesting therefore that age *per se* does not lead to an inevitable decrease in osteocyte density as has been suggested previously by others [571, 448]. Hence, we believe that previous studies reporting age-related decreases in osteocyte density [571, 448], were in fact not determining the effects of age for itself, but the cumulative effects of age and of lack of physical activity on osteocyte viability. From our results, the evidence about the role of physical activity levels as an important aspect in the skeletal changes associated with age comes also from the identification of a significant correlation between several parameters of trabecular bone microarchitecture, particularly in the distal epiphysis, and the distance traveled by the animals in the running wheel. For instance, distal epiphysis BV/TV, Tb.N and Tb.Th were all shown to be positively correlated with the distance traveled by mice, while Tb.Sp was shown to be inversely correlated.

To investigate the effects of the loss of estrogen on bone tissue we used a similar experimental design to that used to investigate the effects of ageing, but using as an animal model the ovariectomized female rat [564] (studies 4 and 5). Again, animals housed with access to the running wheel (OVX+EX) were considered as those reflecting the effects of estrogen loss on bone tissue, while animals housed in standard conditions (OVX+SED) would additionally reflect the consequences of sedentary behavior. Surprisingly, we did not found in OVX+EX animals one of the hallmarks of estrogen loss-induced changes in bone metabolism, which is the significant increase in bone resorption [607]. Our results showed that OVX animals that were maintained in physically active housing conditions following the ovariectomy had only a slightly increased serum concentration of the bone resorption and formation markers compared to sham-operated animals of the same age and housing conditions, which however did not reached statistical significance. In addition, there was also no substantial evidence suggesting highly unbalanced bone turnover in OVX+EX animals. Quite interestingly, results from a previous study [597] provide compelling evidence

suggesting that the overall rate of bone turnover is mostly regulated by estrogen levels, but the balance between bone formation and resorption seem to be mostly modulated by mechanical stimulation. Considering the evidences from this study as well as our results, we believe that the unbalanced bone resorption commonly described following estrogen loss might be in part caused by lack of mechanical stimulation, and not just because of estrogen loss, because otherwise it would be expected to observe a significantly increased bone turnover in OVX+EX animals, which was not the case.

Even though bone turnover was not significantly increased in OVX+EX compared to SHAM+EX animals, differences in bone mineralization degree were detected between these groups but however not between OVX+EX and younger controls (BSL). This finding suggests that with age there was a tendency for bone tissue to become more mineralized, but ovariectomy hindered that increase in bone mineralization degree. Therefore, OVX+EX animals were unable to attain the same degree of bone tissue mineralization as SHAM+EX animals, but however OVX+EX animals did not evidenced a decrease regarding the baseline mineralization degree conditions.

Regarding trabecular microarchitecture, the histomorphometric analysis demonstrated that ovariectomy caused a dramatic trabecular bone mass loss and resulted in a significant deterioration of the trabecular microarchitecture at both epiphyses. In the OVX+EX animal's proximal and distal epiphysis, BV/TV and Tb.N were significantly decreased while Tb.Sp was significantly increased when compared to BSL animals. In addition, despite that the proximal epiphysis Ct.Th was higher in OVX+EX animals compared to BSL, demonstrating that in fact there was an increase with age, Ct.Th was still smaller when compared to that of the proximal femur of SHAM+EX animals, suggesting that estrogen loss hindered the Ct.Th increases in with age in the proximal epiphysis. In the distal epiphysis however age and ovariectomy seemed to induce no changes in cortical bone thickness.

In our fifth study we have also analyzed the effects that low estrogen levels have on the femur, but we have focused our attention here to the changes that occur in the cortical bone from the femur mid-diaphysis. Our results (study 5) were very similar

with our findings regarding the effects of age on the cellular density pattern of the male mice cortical bone (study 2). Our analysis to the cellular density pattern of physically active ovariectomized animals, suggests that the loss of estrogen does not necessarily lead to a decrease in osteocyte density, as we have found no differences between OVX+EX and BSL or between OVX+EX and SHAM+EX. Nevertheless, and like we have also observed in the aged male mice, the number of empty lacunae in OVX+EX was still significantly higher than in BSL controls, demonstrating that with age there was some loss of osteocytes, but not to an extent that implicates a significant reduction in osteocyte density within the bone matrix. However, our results also suggest that the loss of estrogen did not influenced this increase in empty lacunae numbers, and that this increase was mostly associated with advancing age as there were no differences between OVX+EX and SHAM+EX animals. The implications of a significant reduction in the number of viable osteocytes in bone have been described by others and are associated with impaired mechanotransduction [547], alterations in the bone hydration status [593], increases in microcrack accumulation [447] as well as in increased intracortical bone resorption [90]. Interestingly, there is evidence from a previous study [156], that ovariectomized mice have a significant increase in osteocyte apoptosis, which leads to increased osteoclast activation and intracortical resorption with the consequent increase in cortical porosity. Importantly, intracortical porosity has also been shown in some studies to be a hallmark of the ageing skeleton [119, 623] and there is evidence that it significantly compromises cortical bone biomechanical properties [146, 85]. Notoriously, our analysis to the femur mid-diaphysis cortical bone demonstrates that in addition of not leading to a significant decrease in osteocyte density, ovariectomy and age did not also resulted in a significant increase in intracortical bone resorption, as there were no differences between OVX+EX and BSL regarding these parameters. Therefore, our results suggest that the increase in intracortical porosity does not seem to be an inevitable consequence of age and estrogen loss. Regarding our results it is also interesting to consider the findings from a previous study [166] who describes the age-related changes in human femoral cortical bone. The results from this study show that there was a large variance in cortical bone

porosity between individuals of the same age, with bones from aged individuals displaying large increases in porosity when compared with bones from younger individuals while others surprisingly displayed almost no differences. Therefore, these results further strengthen our conviction that some of the age and estrogen loss related features of bone might not result only from age and estrogen loss and that other variables, as most likely the physical activity levels, also play an important role in the evolution of those changes.

In addition of analyzing the cellular density pattern, we have also assayed in this study (study 5) the cortical bone geometry from the femoral mid-diaphysis. There is evidence that the mid-shaft cross section of long bones tends to increase with age [479] and that estrogen loss is also associated with increases in periosteal expansion [499, 14]. In agreement with these findings, our results also showed that the combined effects of age and estrogen-loss induced a significant increase of both the endocortical and periosteal bone dimensions. This effect was evidenced by the significantly higher bone diameter (B.Dm) and marrow cavity diameter (Ma.Dm) observed in OVX+EX animals when compared to BSL. However, the increase in endocortical dimensions was not sufficient enough to lead to a significant decrease in Ct.Th in OVX+EX animals as there were no differences between them and BSL. As periosteal expansion increases the bending strength of the diaphysis [560], this adaptation has been considered to be a compensatory mechanism that allows bone strength to be maintained despite significant decreases in the amount of bone tissue or on the deterioration of its properties [479, 499, 409, 73]. Interestingly, our results also showed that both Young modulus and ultimate stress were inversely correlated with endocortical and periosteal cortical bone dimensions, suggesting therefore that periosteal and endocortical expansion were in fact signs of a decrease in bone tissue mechanical properties.

The proximal femur geometry was also analyzed on study 4, and our results suggest that some alterations were also induced here by estrogen loss. Analysis to the femur length showed that it increased with age in all animals, irrespectively of estrogen loss, which is in accordance to what was expected considering that rat continues to display

some longitudinal bone growth in the adulthood [564]. Nevertheless, increases in femur length were higher in ovariectomized animals when compared to sham-operated counterparts, suggesting therefore that loss of estrogen significantly enhanced bone length increases, which is also in agreement with the findings of a higher periosteal expansion in cortical bone. In addition to the higher femur length, ovariectomized animals also displayed a significantly higher femoral neck axis length (FNAL) and neck-shaft angle (NSA) when compared to both BSL and sham-operated counterparts, suggesting that the combination of age and estrogen loss significantly increased these dimensions in the proximal femur. Interestingly, both femur length and FNAL were shown (in study 3) to be significantly associated with an increase in the femoral neck ultimate strength. There is also evidence from a previous study that increases in FNAL are associated with increases in femoral neck ultimate strength [106]. Therefore, and like in the cortical bone from the diaphysis, these adaptations may represent an adaptive response of the bone to maintain its mechanical strength possibly compensating for decreases in other properties as it has been suggested previously [130]. Indeed our results showed that there were no differences in bone strength between ovariectomized and either BSL or sham-operated animals, which suggests that geometric adaptations might have compensated decreases in other bone properties, thereby contributing to the preservation of overall bone strength.

Several bone tissue alterations that have been associated with age and estrogen loss were shown in our studies to be prevented or attenuated when the animals were housed in the cages with activity wheel. Therefore and as we have previously mentioned, the alterations observed in physically active animals are considered to be the consequence of age, and to reflect what would be the skeletal consequences of age if the animals were in the wild, where they could freely move. Having this in consideration we will now discuss what were the main skeletal changes observed in the animals that were constrained to the cages without running wheel and that therefore besides of ageing and estrogen loss, also suffered the influence of sedentary behavior.

Sedentary behavior enhances some of the bone tissue alterations induced by age and estrogen loss

Histomorphometric analysis performed to the proximal and distal epiphysis of the mice femur (study 2) suggests that the lack of physical activity significantly enhances the age-induced deterioration of the trabecular microarchitecture. Despite that both groups of aged animals (aged active and aged sedentary) had an overall deterioration of the trabecular microarchitecture when compared to younger animals, differences between AA and aged sedentary animals (AS) were also detected. For instance, Tb.N was significantly lower and Tb.Sp was significantly higher in AS compared to AA animals in both epiphyses. Similar results regarding the effects of sedentary behavior and estrogen loss on trabecular microarchitecture were also identified (study 4). Despite that ovariectomy lead to a dramatic loss of trabecular bone, these losses were shown to be significantly more pronounced in animals housed in sedentary conditions than in their physically active counterparts. For instance, in the proximal femur, BV/TV (-65%), Tb.N (-35%) and Tb.Th (-23%) were all significantly lower in sedentary when compared to physically active ovariectomized animals while Tb.Sp (+41%) was significantly higher in sedentary compared to physically active ovariectomized animals. A similar pattern of disuse-enhanced trabecular deterioration was also identified in the distal epiphysis of the femur, with sedentary animals displaying half the BV/TV and an 81% lower Tb.N as well as a 90% higher Tb.Sp than physically active ovariectomized animals. The identification of these differences in trabecular microarchitecture between physically active and sedentary animals therefore suggests that when the animals are housed in standard laboratory conditions they are being constrained to a significant degree of sedentary behavior which greatly enhances the deterioration of the trabecular microarchitecture that is already being affected by age and/or estrogen loss.

The substantial differences in trabecular microarchitecture observed between physically active and sedentary ovariectomized female rats were however not surprising considering the effects that sedentary behavior had on the bone turnover rate and balance in these animals. Indeed, the serum concentration of the biochemical

marker of bone resorption was significantly higher in sedentary than in physically active ovariectomized animals. Nevertheless, bone formation was only slightly, but not significantly, higher in sedentary than in physically active ovariectomized animals. These results therefore suggest that physical inactivity contributed to the unbalance of bone turnover in OVX animals by favoring bone resorption, which is in line with evidence provided in previous studies suggesting that mechanical strain is a key determinant of bone turnover balance in the absence of estrogen [597, 563].

As mentioned previously, increased bone turnover rate is associated with decreases in bone tissue mineralization degree due to the reduction in the time available for secondary mineralization to occur [64]. In fact, we have also identified a significant inverse correlation between the concentration of the bone resorption marker and both calcium and phosphate densities (study 3), which further supports the importance of bone turnover in determining bone mineralization degree. Therefore, by stimulating the increase in bone resorption in ovariectomized animals, sedentary behavior also resulted in a lower bone tissue mineralization degree, as it is evidenced by the lower calcium and phosphate densities in sedentary compared to physically active ovariectomized animals.

Considering therefore that physical inactivity enhanced the deterioration of the trabecular microarchitecture and lead to a decrease in the bone tissue mineralization degree, it was not surprising that we have encountered significant differences in the bone tissue properties between exercised and sedentary ovariectomized animals. In study 3, our results showed that Young modulus was significantly correlated with Tb.Th, bone calcium content and bone phosphate content, and that the combination of these properties was responsible for 34% of the Young modulus variation. Notoriously, all these several bone properties were decreased in OVX animals housed in sedentary conditions when compared to counterparts housed on physically active conditions thereby leading to a significantly lower Young modulus in sedentary animals. Quite interesting though was the finding that ultimate bone strength was not significantly different between sedentary and physically active animals considering the differences encountered in trabecular architecture and bone tissue mineralization

degree. As referred previously, with age and following estrogen loss, the bone tends to suffer an adaptation on its geometry, which seems to be a compensatory response to the decrease in other bone properties [47, 73]. In study 3, we performed multiple regression analysis to investigate the extent to which the proximal femur bone geometry was able to predicted the bone ultimate strength, and it was shown that the combination of FNAL, CFMA, FNW and femur length were responsible for 47% of the proximal femur ultimate strength variation. Interestingly, despite that differences between sedentary and physically active ovariectomized animals only reached statistical significance for FNAL, the mean values of all these parameters were higher in sedentary animals suggesting that their geometric adaptations were more pronounced than in physically active counterparts. These adaptations therefore seem to be responsible for the absence of differences in bone strength between sedentary and physically active OVX animals.

The influence of sedentary behavior was also noticed in the femur mid-diaphysis geometry in our studies with both ovariectomized female rats (study 5) as well as with aged mice (study 2). Following ovariectomy, an expansion of the periosteal diameter was observed in both sedentary and physically active animals. Despite that the mean values of total bone and marrow cavity diameter were slightly higher in sedentary animals, the differences among groups did not reached statistical significance. Nevertheless, the results regarding cortical bone thickness suggest that the expansion of the endocortical diameter in sedentary animals surpassed the increases in periosteal expansion as sedentary animals evidenced a significant decrease in cortical bone thickness with age, an outcome that was however not identified in physically active counterparts.

In our study with aged male mice (study 2), we have also identified that sedentary animals had a significantly higher periosteal (B.Dm) and endocortical (Ma.Dm) bone expansion then their physically active counterparts, which is in agreement with our findings in ovariectomized female rats. Nevertheless, we observed a surprisingly higher Ct.Th in sedentary animals. This finding was quite unexpected as it was anticipated that sedentary behavior would have, like in the female ovariectomized rat, unbalanced

bone turnover towards bone resorption. Despite that we do not have data regarding bone turnover in this study, we speculate that differences in Ct.Th between sedentary and physically active aged mice may be associated with a reduction in bone resorption considering the significantly reduced osteocyte density that was observed in aged sedentary animals and the existence of evidence suggesting that the integrity of the osteocyte network is a requirement for bone resorption to occur [510, 547].

In ovariectomized female rats, sedentary behavior was also shown to have a profound effect on the pattern of bone tissue cellular density (study 5), with OVX animals housed in sedentary conditions displaying a significant reduction in osteocyte density with age, and a significantly lower osteocyte density than their physically active counterparts. Moreover, despite that the number of empty lacunae increased in both groups of OVX animals with age, increases were considerably higher in sedentary animals suggesting therefore that physical inactivity played a pivotal role in determining osteocyte viability.

There is compelling evidence suggesting that osteocyte death stimulates the activation of osteoclasts [301, 151, 290] which leads to the increase in local bone resorption [90]. If restricted to focal bone regions, this resorption constitutes a protective mechanism that promotes the renewal of damaged bone [90]. However large increases in osteocyte death could lead to excessive bone resorption and increased intracortical porosity, ultimately compromising bone strength [547]. Interestingly our results also showed that in sedentary animals intracortical bone porosity was substantially higher than in physically active counterparts, and that this increase in cortical porosity was significantly correlated with increases in empty lacunae number. These results therefore demonstrate that sedentary behavior was the main factor associated with the loss of osteocytes and with the increase in intracortical bone resorption. Moreover, physical inactivity was also shown to contribute significantly to the decrease in the femur mechanical properties as both the number of empty lacunae and intracortical bone porosity were inversely correlated to the bone ultimate stress. Therefore, biomechanical testing of the femur diaphysis also showed

that sedentary animals had a significantly lower Young modulus and bone ultimate stress when compared to their physically active counterparts.

These several findings therefore suggest that sedentary behavior imposed by standard housing conditions exerted a determinant effect over the bone changes that occur with age and following estrogen loss.

2. Overall Limitations

Bearing in mind the overall experimental design, we consider that it is important to highlight some potential limitations of our studies.

Although the ovariectomized female rat has been widely used as an experimental model for the study of estrogen induced bone loss [256, 318] it still has some drawbacks that are important to mention. Event thought that most of the rat´s bone elongation occurs during the initial third of their lifespan [564], they still continue to display some bone growth throughout adulthood, which however does not happen in humans. Therefore, it is possible that the mechanisms leading to the adaptive response in bone geometry that we observed in rats, might not be so meaningful in humans which may therefore preclude generalization. Another important particularity in rodent's bones, especially in mice, is the existence of minimal intra-cortical bone remodeling due to their ill developed Haversian system. Therefore, it is possible that the effects of physical inactivity on the increase in intracortical porosity may have been at some extent undervalued by our animal models as it may be higher in humans who display considerably higher levels of Haversian remodeling.

It is also important to bear in mind that the ovaryes produce not just estrogen, but also other hormones such as progesterone and testosterone, both of which are also known to influence bone mass [445, 269]. Thus, and despite the widespread use of the ovariectomized female rat as a model for the study of post-menopausal bone loss, it is perhaps not correct to ascribe to the estrogen loss all the skeletal outcomes of the bilateral ovaries removal, as it is possible that other hormones may also be involved.

3. Main Conclusions

Considering the overall findings of our studies the major conclusions of the present work are that:

- Ovariectomy results in a notorious and permanent decrease in voluntary physical activity levels in female rats;
- Decreases in skeletal muscle fibers cross sectional area in ovariectomized female rats appear to result mostly from decreases in voluntary physical activity levels and not from the direct effects of estrogen on skeletal muscle fibers;
- In spite of not experiencing such a dramatic decrease in physical activity levels as female rats following estrogen loss, male mice voluntary physical activity levels are also shown to decline with age;
- Although age and hypoestrogenemia were *per se* major factors leading to trabecular bone loss, sedentary behavior significantly enhanced the deterioration of the proximal and distal femur trabecular microarchitecture;
- Physical inactivity was a major factor leading to unbalances in bone turnover contributing thereby to a decrease in bone tissue mineralization degree in sedentary animals;
- Age and estrogen loss were not shown to lead *per se* to a significant decrease in bone tissue osteocyte density, as a significant reduction in osteocyte density with age was only observed in sedentary animals and not in physically active animals;
- The increase in bone tissue empty lacunae density was associated with increases in intra-cortical bone porosity and reduced cortical bone mechanical properties;

In view of the initial objective of this work our overall conclusion is that even though there is an overall decrease in bone tissue quality with age and following estrogen loss, physical inactivity plays here a major factor, significantly enhancing that deterioration.

4. Perspectives for future research

One of the most interesting findings of our studies was the identification of the protective effects that regular physical activity had on osteocytes, enabling to preserve the osteocyte network in the elder male mice as well as in ovariectomized female rats. This was further shown to have notorious consequences on bone mechanical properties. Regardless of this observation, the mechanisms whereby regular physical activity improved osteocyte viability are to a great extent unknown, especially *in vivo*. We therefore believe that future research should be directed to the identification of the mechanisms that might be involved in this protective effect. Moreover, there is evidence that excessive osteocyte death is also associated with decreases in bone strength and increased fracture risk following the exposure to glucocorticoids [399, 437] and radiation [335, 394] and possibly also following antimetastatic drug administration [528]. Considering our results about the effects of regular physical activity on osteocyte viability, it should be the aim of future research to determine if regular physical activity was also able to prevent decreases in osteocyte loss in these situations.

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