Exercise training, Bone and Aging.
Evidence from different impact loading exercise interventions on age-related bone loss.

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KEY-WORDS: AGE, PHYSICAL ACTIVITY, BONE DENSITY, FRACTURE RISK, BIOMARKERS, META-ANALYSIS.

Old age is like everything else. To make a success of it, you've got to start young.

Fred Astaire

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Abstract

Aging is generally associated with a gradual loss of physiological functions, of muscle mass, strength and balance, and a decrease in bone mineral density (BMD), among other age-related alterations. Increasing evidence supports that exercise has a prominent role on bone health and it may be an interesting strategy to reduce the incidence of bone fractures. However, the optimal training type to elicit both the greatest improvements in BMD, muscle strength and balance and a concomitant alteration of bone-related biomarkers production needs to be explored further. Based on the accumulated evidence from both laboratory and clinical studies, the aims of this thesis were: 1) to examine the relationships between patterns of adipose and lean tissue deposition and BMD in older women, 2) to analyze the effect of different mechanical loading protocols [weight-bearing multicomponent exercise (ME), resistance exercise (RE), aerobic exercise (AE) and RE combined with ME (CE)] on BMD and bone turnover, functional capacity, and inflammatory response in older adults, 3) to meta-analyze the impact training (mixed loading exercise programs) effects on BMD in older adults. To accomplish these goals 4 experimental studies were conducted involving (on total) 155 women and 23 men, and 1 systematic review and meta-analysis that included 1577 older adults (age >60 years). Taken together, outcome measures included lumbar spine (LS) and proximal femur BMD, muscle strength, balance, body composition, biochemical markers of bone remodelling and inflammation. Secondary variables included dietary intake, accelerometer-based physical activity, and molecularly defined lactase nonpersistence. Cross-sectional data revealed that appendicular fat mass had strong and independent associations with femoral neck BMD (26.4% of variance); Longitudinal data shown that after 8 months of training RE group exhibited increases in BMD at the trochanter (2.9%) and total hip (1.5%), ME group increased BMD at the femoral neck (2.8%), and CE group (both men and women) improved BMD at several bone sites. All training groups improved balance, whereas RE training was more effective than AE and ME for inducing favourable changes in muscle strength. Both RE and ME improved body composition. No significant changes were observed in osteoprotegerin (OPG) and receptor activator of nuclear factor-kappaB ligand (RANKL) levels, and OPG/RANKL ratio after RE, AE and CE training. Bone turnover markers also remained unchanged after 8-month CE, while training resulted in a significant reduction of interleukin-6 (only in men), interferon-γ, and C-reactive protein in both men and women. The systematic review and meta-analysis of 19 studies supported the view that exercise of mixed loading is associated with significant increases in femoral neck and LS BMD in older adults. Taken together, results suggest that exercise training that includes odd-impact loading in addition to RE training may confer advantage to improve body composition, inflammation, BMD and reduce fall risk in older adults by increasing balance and muscle strength.

Key words: AGE, PHYSICAL ACTIVITY, BONE DENSITY, FRACTURE RISK, BIOMARKERS, META-ANALYSIS

Resumo

O envelhecimento está normalmente associado, entre outras alterações, a uma perda gradual de funções fisiológicas, perda de massa muscular, força e equilíbrio, e diminuição da densidade mineral óssea (DMO). Estudos sugerem que o exercício físico tem um papel preponderante na saúde óssea, podendo ser considerado uma estratégia para reduzir a incidência de fraturas ósseas. Contudo, o melhor tipo de treino passível de induzir as maiores alterações na DMO, força muscular e equilíbrio, e uma concomitante alteração na produção de biomarcadores associados à remodelação óssea necessita maior esclarecimento. Assim sendo, e tendo por base a evidencia acumulada proveniente de estudos laboratoriais e clínicos, os objetivos da presente tese foram: 1) examinar as relações entre os padrões de distribuição de tecido adiposo e magro e a DMO em mulheres idosas, 2) analisar o efeito de diferentes protocolos com carga mecânica [treino multicomponente com sustentação de peso (TM), treino de força (TF), treino aeróbio (TA), e TF combinado com TM (TC)] na DMO e na remodelação óssea em idosos, 3) meta-analisar os efeitos de exercício com impacto (programas de exercício com diferentes tipos de sobrecarga) na DMO do colo do fémur e da coluna lombar (CL) em idosos. Para atingir estes objetivos foram efetuados 4 estudos experimentais envolvendo (na totalidade) 155 mulheres e 23 homens, e 1 revisão sistemática e meta-análise que incluiu 1577 idosos (idade> 60 anos). Na globalidade, as variáveis em análise incluíram: DMO da CL e do fémur proximal, força muscular, equilíbrio, composição corporal, biomarcadores de remodelação óssea e inflamação. As variáveis secundárias incluíram a análise do consumo nutricional, atividade física diária por acelerometria e resistência à lactose. Os resultados do estudo transversal revelaram que a massa gorda apendicular tem uma forte e independente associação com a DMO do colo do fémur (26,4% da variância); os estudos longitudinais revelaram que após 8 meses de treino o grupo TF exibiu um aumento na DMO do trocanter (2,9%) e da anca (1,5%), o grupo TM aumentou a DMO do colo do fémur (2,8%), e o grupo TC (mulheres e homens) melhorou a DMO em diferentes zonas ósseas do fémur proximal. Todos os grupos de treino melhoraram o equilíbrio, enquanto o TF foi mais eficaz que o TA e o TM na indução de alterações positivas na força muscular. Ambos os grupos TF e TM melhoraram a composição corporal. Não se observaram diferenças significativas nas concentrações séricas de osteoprotegerina (OPG) e do ligante do receptor ativador do fator nuclear-kappaB (RANKL), e do rácio OPG/RANKL após TF, TA e TC. Apesar de os marcadores de remodelação óssea permaneceram inalteráveis, o treino resultou numa redução significativa da interleucina-6 (apenas nos homens), interferon-γ e na proteína C reativa em ambos os géneros. A revisão sistemática e meta-análise de 19 estudos suportou a noção de que programas de exercício com diferentes tipos de sobrecarga induzem aumentos significativos na DMO do colo do fémur e da CL em idosos. Deste modo, os resultados sugerem que programas de treino que incluam sobrecarga mecânica inabitual juntamente com TF podem conferir vantagem para a melhoria da composição corporal, DMO, inflamação e redução do risco de quedas em idosos, através da melhoria do equilíbrio e força muscular.

Palavras-chave: ENVELHECIMENTO, ATIVIDADE FÍSICA, DENSIDADE ÓSSEA, RISCO DE FRATURA, BIOMARCADORES, META-ANÁLISE

List of abreviations

30sCST	¦ 30-second chair-stand test
6MWT	¦ 6-minute walk test
8ft UG	¦ 8-foot Up and Go Test
ACSM	¦ American College of Sports Medicine
AE	¦ Aerobic exercise
AFM	¦ Appendicular fat mass
ANOVA	¦ Analysis of variance
AP	¦ Anterior–posterior
AR	¦ Androgen receptor
B-ALP	¦ Bone specific alkaline phosphatase
BMD	¦ Bone mineral density
BMI	¦ Body mass index
BMSCs	¦ Bone marrow stromal cells
BMU	¦ Basic multicellular unit
BRU	¦ Bone-remodeling unit
BTMs	¦ Bone turnover markers
BUA	¦ Broadband attenuation
BW	¦ Body weight
CE	¦ Combined exercise
CIs	¦ Confidence intervals
CL	¦ Coluna lombar
CON	¦ Control
COP	¦ Center of pressure
CT	¦ Computed tomography
CTX	¦ C-terminal telopeptide of Type I collagen
CV	¦ Coefficients of variation
D	¦ Raw (unstandardized) difference in means
DMO	¦ Densidade mineral óssea
dNTP	¦ Deoxynucleotide triphosphate

DPA	¦ Dual photon absorptiometry
DPD	¦ Deoxypyridinoline
d-wk	¦ Days-week
DXA	¦ Dual energy x-ray absorptiometry
EA	¦ Elliptical area
ELISA	¦ Enzyme-linked immunosorbent assay
ER	¦ Estrogen receptor
ES	¦ Effect size
ET	¦ Exercise-training
FEA	¦ Finite element analysis
FM	¦ Fat mass
GRFs	¦ Ground reaction forces
H	¦ High
HR-MRI	¦ High-resolution magnetic resonance imaging
Hs-CRP	¦ High sensitive C-reactive protein
I^2	¦ I-squared
ICTP	¦ C terminal telopeptide
IGF-1	¦ Insulin-like growth factor 1
IL	¦ Interleukin
Inter	¦ Intertrochanteric region
IFNs	¦ Interferons
ITT	¦ Intention-to-treat
KE	¦ Knee extension
KF	¦ Knee flexion
L	¦ Left
L	¦ Low
LM	¦ Lean mass
LS	¦ Lumbar spine
M-CSF	¦ Macrophage colony-stimulating factor
ME	¦ Multicomponent exercise
MES	¦ Minimum effective strain
ML	¦ Medial–lateral
MRI	¦ Magnetic resonance imaging
MVPA	¦ Moderate to vigorous physical activity
NAMS	¦ North American Menopause Society

NTX	¦ N-terminal telopeptide of Type I collagen
OC	¦ Osteocalcin
OLS	¦ One-leg stance
OPG	¦ Osteoprotegerin
PA	¦ Physical activity
PCRs	¦ Polymerase chain reactions
PICP	¦ Procollagen I C terminal extension propeptide
PINP	¦ Procollagen I N terminal extension propeptide
pQCT	¦ Peripheral quantitative computed tomography
PRAL	¦ Potential renal acid load
PT	¦ Peak torque
PTH	¦ Parathyroid hormone
QCT	¦ Quantitative computed tomography
QUS	¦ Quantitative ultrasound
R	¦ Right
RANKL	¦ Receptor activator of nuclear factor kappaB ligand
RCTs	¦ Randomized controlled trials
RE	¦ Resistance exercise
ROI	¦ Region of interest
RVT	¦ Rotational vibration training
SD	¦ Standard deviation
SE	¦ Standard error
SERM	¦ Selective estrogen receptor modulator
SNP	¦ Single nucleotide polymorphism
SNR	¦ Signal-to-noise ratio
SOS	¦ Speed of sound
SPA	¦ Single photon absorptiometry
τ^2	¦ Tau-squared
TA	¦ Treino aeróbio
TC	¦ Treino combinado
TF	¦ Treino de força
TC	¦ Tai Chi
TM	¦ Treino multicomponente
TNF	¦ Tumor necrosis factor
TRACP	¦ Tartrate resistent acid phosphatase

Troch	¦ Trochanter
V	¦ Variance
Vib	¦ Vibration
vQCT	¦ Volumetric quantitative computed tomography
VVT	¦ Vertical vibration training
WHO	¦ World Health Organization
WMD	¦ Weighted mean difference

GENERAL INTRODUCTION

General introduction

Peak bone mass is attained during the third decade of life. Subsequently, bone mass declines with advancing age, causing bone fragility, which lead to the so-called “senile osteoporosis”. This bone loss, which should not be understood as synonymous of resorptive removal of bone, as it also represents the failure of bone formation, is the net result of the amount of bone resorbed on the endosteal surfaces and the amount formed in the periosteal surface. Thus, age-related bone loss can be seen as a result of an alteration in bone turnover, which becomes progressively attenuated (Duque & Troen, 2008). The increasing longevity of modern populations and recent technological advances trigger an explosion of interest in the area of osteoporosis prevention and treatment. It has been estimated that the number of hip fractures will double to 2.6 million by the year 2025, with a greater percentage increase in men than in women (Gullberg et al., 1997). Decreasing the prevalence of osteoporotic fracture and public health burdens will certainly depend on the possibility to modify the genetics of bone biology and/or other mechanisms underlying bone loss and neuromuscular degenerations. Many pharmacological and non-pharmacological preventive and therapeutic strategies have been explored to counteract age-related bone loss. These include selective estrogen receptor modulator (SERM), bisphosphonates, calcitonin, calcium and vitamin D supplementation, and physical activity and exercise recommendations ("Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society", 2010). In this context, exercise is viewed as the only single therapy that can simultaneously target, not only the attenuated bone turnover throughout the old age, but also augment muscle mass, muscle strength, balance, and bone strength and decrease the risk of falling (Carter et al., 2001). There is definitive evidence that bone senses and adapts to mechanical stimuli (changing mechanical loads) and early observations on this subject were pointed out long time ago by Galileo (1638), although Julius Wolff had routinely received the credit for this idea. Clearly, scientific advances have been developed since then, despite human studies have not always found results in line with mechanical loading experiments using animal models and *in vitro* setups (Zernicke et al., 2006). In fact, several studies have examined the effects of chronic exercise on

bone mass across ages, but pre and postmenopausal women have received a disproportional attention due to the impact of the cessation of estrogen production around the menopause on bone health (Weitzmann & Pacifici, 2006). Thus, to date, less is known regarding the variances of bone response to mechanical loading in older adults (Chodzko-Zajko et al., 2009; Kohrt et al., 2004), and which type of skeletal loading would account for the higher beneficial changes in bone mass and also in decreasing risk of falling. Moreover, quantitative changes in skeletal turnover detected by bone metabolism biomarkers may provide complementary and sensitive data on the current metabolic status of the bone that resulted from therapeutic interventions. However, only few studies focused on older adults have addressed the influence of prolonged exercise on these biochemical markers (Bemben et al., 2010; Vincent & Braith, 2002a).

Although falls occur as a result of a complex interaction of risk factors, some potential modifiable factors are amenable to changes in old age, such as decline of physical capacities, balance and muscle strength, included in the biological risk factors category (Dontas & Yiannakopoulos, 2007). Several previous studies have examined the influence of exercise interventions on fall prevention (de Kam et al., 2009). Generally, studies had focused on frail participants, with reduced physical function (Karinkanta et al., 2007), and interventions were designed targeting balance and muscle strength improvement, apart of bone changes (Ballard et al., 2004; Buchner et al., 1997; Day et al., 2002). Thus, little evidence exist on the preventive effect of exercise on older adults with no severe balance disorders, and which type of exercise prescription is more effective on improving fall risk factors and bone mineral density (BMD), as both are strongly related to bone fracture risk (Peeters et al., 2009). Also, more constraints are involved in the exercise prescriptions for this age group, including physical impairments, chronic conditions, and requirement of activities with low risk of falling.

Other potential factors in the maintenance of optimal bone health include lifestyle factors, comprising nutrition and daily physical activity. Until recently, focus has primarily been placed on calcium, vitamin D and a few isolated nutrients. Moreover, most previous studies (Tucker et al., 1999; Tucker et al., 2001) concerning the association between dietary calcium and BMD have not considered the potential impact of lactase nonpersistence, which has been associated with reduced intake of calcium (Bacsi et al., 2009), bone loss (Laaksonen et al., 2009) and bone density (Obermayer-Pietsch et al., 2004), and could represent a genetic risk factor for bone fractures for older adults (Enattah et al., 2005). In addition, physical inactivity has

consistently been pointed out as a modifiable factor contributing to low BMD, osteoporosis and increased risk of fractures (Kumar et al., 2010; Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society", 2010). However, (i) a large body of literature to date only includes physical activity as a potential confounding variable, (ii) the used methods to assess physical activity have poor reliability (Beck et al., 2009; Travison et al., 2008), and (iii) some studies have no data regarding physical activity status. Consequently, using direct measures to assess specific factors that influence BMD has become increasingly important for optimizing appropriate intervention strategies and for reinforcing the accruing body of literature.

Although in the last two decades large efforts have been made (i) on preventive strategies and treatment (Grossman, 2011), (ii) on discern pathogenetic pathways on a molecular and cellular level (Boyle et al., 2003; Scott et al., 2008), and (iii) on defining the physiology of skeletal remodeling in the elderly (Kohrt, 2001), there are few human longitudinal studies addressing the effect of exercise impact interventions on bone density, bone turnover, balance and strength (as biological risk factors for falls in older age). Moreover important confounding variables, including fat and lean mass, daily physical activity, dietary nutrition and lactase nonpersistence, that may also modulate the magnitude of changes, were not always considered in previous studies. Thus, the main purpose of the present work was to analyze the role of different impact loading protocols against fracture risk factors and bone-related biomarkers in older humans, contributing to better understand the preventing and therapeutic effect of exercise on the aging skeleton.

This general objective supported the specific goals of the original articles corresponding to the four chapters of the experimental work of this thesis and a chapter devoted to a systematic review and meta-analysis, specifically:

Paper I

To examine the relationships between patterns of adipose and lean tissue deposition and BMD in older women, independently of age, age at menopause, height, physical activity, muscle strength, nutritional intake, and acid-based load.

Paper II

(i) to assess the effects of an 8-month program of multicomponent training with weight-bearing exercises on different risk factors of falling, such as muscle strength, balance, agility, and BMD, in community-dwelling older women;
(ii) to analyze the co-influence of the confounding variables such as diet, physical activity levels, and lactase persistence;
(iii) to relate these findings with the mechanical loading [using ground reaction forces (GRFs) data] associated with the weight-bearing exercises.

Paper III

(i) to compare the alterations in key factors associated with fracture risk, namely BMD, muscle strength and balance.
(ii) to investigate the expression of serum levels of osteoprotegerin (OPG), receptor activator of nuclear factor kappaB ligand (RANKL) and their ratio after two long-term (8 months) exercise training programs (resistance exercise and aerobic exercise).

Paper IV

(i) to compare the alterations in balance as a key factor associated with fall risk and on BMD, in older men and women after an 8-month combined impact loading intervention.
(ii) to analyze the exercise effect on bone-related inflammatory cytokines, and different biomarkers of bone metabolism.

Paper V

To assess the effects of exercise interventions with different impact loading characteristics on lumbar spine and femoral neck on BMD in older adults by a systematic review and a meta-analysis of randomized controlled trials (RCTs).

In order to achieve these objectives, this thesis was organized in five chapters; in the following (**chapter 1**), the specific topics related to bone health that provide a brief background to our experimental work were summarized. Eleven themes were presented, comprising: the cellular mechanisms of age-related bone loss, the

determinants of bone loss in elderly, the link between inflammation and bone loss, the nutritional mechanisms of age-related bone loss, calcium and vitamin D and the aging skeleton: efficacy in the treatment of osteoporosis, genetic lactase nonpersistence (C/T-13910 polymorphism) and bone health, osteoporosis and fragility fractures in the elderly, risk factors for falling: role of exercise in fall prevention, the adaptive bone response to mechanical loading, measuring bone properties, and finally we reviewed the biochemical markers of bone turnover in osteoporosis. **Chapter 2** was devoted to the experimental work, which includes the papers published (4) or submitted (1) to peer-reviewed scientific journals. The main findings, overall and methodological discussion are presented on **chapter 3**, and on **chapter 4** the main conclusions of the present thesis were clarified. Finally, the references that supported the current scientific work are depicted on **chapter 5**.

[1] THEORETICAL BACKGROUND

Cellular mechanisms of age-related bone loss

Bone remodeling, the process whereby bone is consistently renewed during adulthood, is a homeostatic function (the destruction of bone by osteoclasts followed by *de novo* bone formation by osteoblasts) that allows repair of micro- and macro-damages and maintenance of bone mass and of the structural properties of the skeleton (Hill, 1998). The remodeling sequence involves focally organized cellular events termed bone-remodeling unit (BRU) or basic multicellular units (BMU) (Parfitt, 2002) (Figure 1).

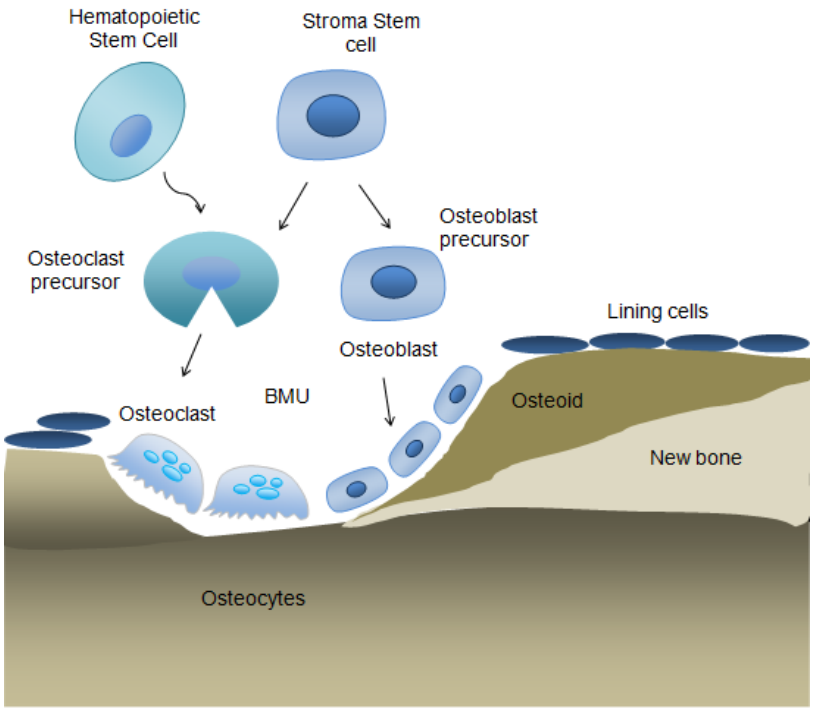


Figure 1 – Schematic model of bone remodeling sequence (sequential action of osteoclasts and osteoblasts to remove old bone and replace it with new bone).

However, changes in the biology of bone cells, with direct consequences on bone turnover can be traced to the effects of aging. Age-related bone loss can be seen as the result of an alteration in bone remodeling and bone turnover. With aging, the

process of bone turnover becomes attenuated (Eriksen, 1986). Consequently, the half-life of the bone matrix is extended, due to its exposure to fatigue-induced damage and microfracure, resulting in inferior material properties (Parfitt, 1993). In addition, turnover becomes unbalanced such that the amount of deposited bone is less than the removed. There are several possible scenarios by which both the attenuation of bone turnover and the lack of complete filing can occur, mediated by osteoclastic cells, osteogenic cells, or both.

The early postmenopausal years are associated with accelerated bone loss, which may be attributed to the increased expression of some bone-resorbing cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), macrophage colony-stimulating factor (M-CSF), and prostaglandins as potential candidates for mediating the bone loss following estrogen deficiency (Khosla & Riggs, 2005). There is an increased rate of remodeling and tilts the balance between bone resorption and formation in favor of resorption, leading to bone loss by alteration of bone cell production and by prolonging osteoclast lifespan and shortening osteoblast lifespan (Jilka, 2003).

It is clear that aging has a major impact on the structure and function of bone marrow that contains the progenitors of both osteogenic and osteoclastic cells (Manolagas & Parfitt, 2010). However, with aging there is a shift in the balance from a stroma that actively supports osteogenesis and exuberant hamatopoiesis to one that is primarily adipogenic. Thus, the main changes that occur on bone formation and resorption due to aging can be summarized as follows:

1) Regarding bone formation, a number of studies point to the possibility that the osteogenic potential of the bone marrow stroma diminishes with age (Weinstein & Manolagas, 2000). Bone marrow stromal cells (BMSCs) are also affected by changes in the balance of systemic factors (such as parathyroid hormone (PTH), vitamin D metabolites, sex steroids, growth factors, and cytokines, which are likely to change in aging) that alter bone metabolism (Manolagas & Jilka, 1995). It is also clear that the aging cell has altered responsiveness to external signals, perhaps through changes in receptor content, receptor activity, intracellular signaling pathways, and regulation of gene expression (Robey & Bianco, 1999). There are factors intrinsic to stromal cells that change with age. These include their lack of self-renewal (decrease in number of precursor cells in the marrow stroma), decreased rate of cell proliferation, decreased cellular activity (reduced responsiveness, reduced production of bone matrix proteins, and other cell products), and the apparent loss of plasticity (inability to convert from one phenotype to another) (Robey & Bianco, 1999).

2) Regarding bone resorption, there may be decreases in the level of peripheral blood stem cells (Egusa et al., 1998) and in the hematopoietic reserve capacity (Balducci et al., 2005; Lipschitz, 1995) as a function of age. The activity of hematopoietic cells, such as macrophages, also varies with aging (Faust et al., 1997; Shepard & Zon, 2000). An impaired ability of stromal cells to support the differentiation of certain types of blood cells which are responsible for stimulating the generation of hematopoietic components ocurrs (Buchanan et al., 1996). Because members of the BMSCs lineage are responsible for the generation of osteoclasts, the combined effects of extrinsic and intrinsic factors that impinge on stromal cell activity as a function of aging also impinge on their ability to stimulate osteoclast formation (Balducci et al., 2005).

It has been postulated that in age-related bone loss, osteoclast are more active *per* cell basis and remove more bone during a cycle of resorption (Eriksen, 1986). Prevailing evidence strongly suggests that it is primarily the reduced osteoblastic activity and number (inability of BMSCs to deposit adequate amounts of bone to compensate for the amount removed by osteoclasts) that lead to decreased bone mass in the aging skeleton (Manolagas & Jilka, 1995).

Determinants of bone loss in elderly

Improving BMD trough a significant decrease in the rate of bone loss is a common goal for people with low bone mass, particularly postmenopausal women and older adults. The fundamental determinants of bone loss have been systematically studied across the spectrum of potentially risk factors. In addition to age, the major factors that influence bone mass are genetics, lifestyle, and menopausal status ("Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society", 2010).

It is well established that BMD is under a strong genetic control. Heredity plays a determinant role on peak bone mass acquisition (i.e., the maximal BMD gained during the skeletal development and maturation phase), accounting up to 80-90% in its variability (Duncan & Brown, 2010; Ralston & de Crombrugghe, 2006). The genetic component of osteoporosis is determined by an assembly of multiple genes with small individual effects, each gene most likely responsible for less than 5% of the genetic variance in the general population (Kung & Huang, 2007). Both linkage (Family-based linkage studies) and association (Candidate-gene association studies) methods have

been used to identify genetic susceptibility *loci* to osteoporosis (Duncan & Brown, 2010).

Several lifestyle factors associated with the risk of low BMD have been identified including poor nutrition, insufficient physical activity, cigarette smoking, and heavy alcohol consumption (Kung & Huang, 2007; Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society", 2010).

A balanced diet modulates bone development and the maintenance of bone health throughout life (Cashman, 2007). As older adults have commonly deficient diets, because of inadequate consume of the recommended servings of dairy products, fruit, vegetables, or grains, nutrition plays a crucial role in this population (Milaneschi et al., 2010; Morley, 2001) (for a complete description of nutritional factors, see chapter 1 on “Nutritional mechanisms of age-related bone loss” pp. 40).

Being thin and/or having a body mass index (BMI) less than 20 kg/m^2 are risk factors for low BMD (De Laet et al., 2005). It is well established that body weight is related to BMD (Reid, 2010) and change in body weight is also a predictor of bone alterations (Shapses & Riedt, 2006). Body weight is largely made up of two components: fat mass (FM) and lean mass (LM). Similarities between obesity and osteoporosis have been identified, suggesting some type of pathologic linkage (Cao, 2011; Rosen & Bouxsein, 2006). Conversely, obese individuals are thought to have a reduced risk for osteoporosis (Bacon et al., 2004), potentially due to increased skeletal loading and increased concentrations of certain hormones, such as estradiol in women (Rosen & Bouxsein, 2006). In addition to the relationship with bone, obesity is associated with low-grade systemic inflammation which is related to an increased risk of type 2 diabetes and cardiovascular disease (Fantuzzi, 2005) but also osteoporosis (Pfeilschifter et al., 2002). Based on the current state of knowledge, it is unclear whether fat has beneficial effects on bone (Zhao et al., 2008). Moreover, the relative contribution of FM and LM to the variation in BMD (Ho-Pham et al., 2010) is presently highly contentious.

Regular exercise has been associated with increased BMD, by positively changing bone turnover in favor to bone formation. There is general agreement that weight-bearing exercise and high-impact exercises provide a positive osteogenic stimulus (Kohrt et al., 2004) (for a complete description of exercise impact on bone loss, see chapter 1 on “Adaptive bone response to mechanical loading” pp. 51).

Chronic alcoholism leads to lower BMD and higher fracture risk due to a combination of factors: 1) poor nutrition and malabsorption of critical nutrients, particularly calcium, magnesium and zinc; 2) liver disease, abnormal vitamin D metabolites and parathyroid function; and 3) direct toxicity to osteoblasts (Ilich & Kerstetter, 2000). It is believed that cigarette smokers may have impaired calcium absorption (Krall & Dawson-Hughes, 1999) although the exact mechanisms by which smoking might adversely affect bone mass are unknown. Compared with nonsmokers, women smokers tend, on average, to lose bone more rapidly, have lower bone mass, and reach menopause 2 years earlier (Kato et al., 1998).

The increased rate of bone resorption immediately after menopause clearly indicates a hormonal influence on bone density in women. Clearly, loss of estrogen leads to increased rate of remodeling and tilts the balance between bone resorption and formation in favor of the former (Frenkel et al., 2010). Classical receptors for estrogens (ERα and ERβ) or androgens (AR) are present in chondrocytes, BMSCs, osteoblasts, and osteoclasts and their progenitors, indicating that the effects of sex steroids on bone are mediated, at least in part, directly (Frenkel et al., 2010). Thus, it is now believed that loss of estrogens and androgens stimulate both osteoclastogenesis and osteoblastogenesis, and they have a critical role on osteocytes apoptosis (Manolagas et al., 2002).

Finally, diverse medications, disease states, and genetic disorders are associated with bone loss, which are categorized as secondary causes of bone loss, as illustrated in table 1.

Table 1 – Secondary causes of bone loss *(Adapted from 2010 NAMS position statement)*

MEDICATIONS	Aromatase inhibitors / Cytotoxic agents / Excessive thyroxine doses/ Gonadotropin-releasing hormone agonists or analogues / Heparin / Immunosuppressives (e.g., cyclosporine) / Intramuscular medroxyprogesterone / Long-term use of certain anticonvulsants (e.g., phenytoin) / Long-term use of oral or intramuscular glucocorticoids
GENETIC DISORDERS	Hemochromatosis / Hypophosphatasia / Osteogenesis imperfect / Thalassemia
DISORDERS OF CALCIUM BALANCE	Hypercalciuria / Vitamin D deficiency
ENDOCRINOPATHIES	Cortisol excess / Cushing's syndrome / Gonadal insufficiency (primary and secondary) Hyperthyroidism / Primary hyperparathyroidism / Type 1 diabetes mellitus
GASTROINTESTINAL DISEASES	Billroth I gastroenterostomy / Chronic liver disease (e.g., primary biliary cirrhosis) Malabsorption syndromes (e.g., celiac disease, Crohn's disease) / Total gastrectomy
OTHER DISORDERS AND CONDITIONS	Ankylosing spondylitis / Chronic renal disease / Lymphoma and leukemia / Multiple myeloma / Nutritional disorders (e.g., anorexia nervosa) / Rheumatoid arthritis / Systemic mastocytosis

Despite the compelling evidence mostly extrapolated from cross-sectional studies, some studies failed to demonstrate a clear influence of all the previously described risk factors. A recent systematic review (Papaioannou et al., 2009) showed that advancing age, smoking, and low weight/weight loss were consistent risk factors for bone loss in older men. Although less evidence was available, physical/functional limitations and prevalent fracture (after age 50) were also associated with low BMD/bone loss (Papaioannou et al., 2009). Moreover, evidence was inconsistent or weak for physical activity, alcohol consumption, calcium intake, muscle strength, family history of fracture or osteoporosis, and height or height loss (Papaioannou et al., 2009). Data from a 4-year longitudinal study on risk factors for change in BMD in older adults showed that risk factors consistently associated with bone loss include female sex, thinness, and weight loss, while weight gain appears to protect against bone loss (Hannan et al., 2000). In addition, data suggested that current estrogen use may help to maintain bone in women, whereas current smoking was associated with bone loss in men. Surprisingly, bone loss was not affected by caffeine, physical activity, serum vitamin D, or calcium intake (Hannan et al., 2000).

Link between inflammation and bone loss

Evidence continues to accumulate that factors involved in inflammation are linked with those critical for bone physiology and remodeling (Arron & Choi, 2000; Lorenzo, 2000). The effect of inflammation on bone is mediated by pro-inflammatory cytokines, which regulate bone formation as well as bone resorption thereby altering bone homeostasis (Schett, 2011).

Cytokine-mediated bone damage is primarily driven by the effects of these mediators on the differentiation and activity of the bone-resorbing cell, the osteoclast (Schett, 2011). Osteoclasts are hematopoietic cell stemming from the monocyte linage, which undergo a series of differentiation steps to become mature bone-resorbing cells (Boyle et al., 2003). Several pro-inflammatory cytokines (such as TNFα, IL-1, IL-6 and IL-17) are major triggers for osteoclast activation explaining the enhanced bone loss during inflammation (Schett, 2011). On the other hand other cytokines such as IL-12, IL-18,

IL-33 and interferons (IFN) are strong suppressors of osteoclast differentiation and inhibit bone loss (Schett, 2011).

In the following, the effects of key cytokines (RANKL, OPG, TNF, IL-6, IFNs, and CRP) on osteoclastogenesis and bone resorption will be summarized. In brief, some of these osteoclast-inducing cytokines stimulate osteoclast differentiation directly, whereas others support osteoclast differentiation indirectly through acting on nonosteoclast lineage cells.

RANKL and M-CSF, also designated CSF-1, are the two essential cytokines for basal osteoclastogenesis (Boyle et al., 2003; Pixley & Stanley, 2004). RANKL, a member of TNF superfamily, is the key osteoclastogenic cytokine, because osteoclast formation requires its presence or its priming of precursor cells. OPG is a decoy receptor for RANKL and can block RANKL/RANK interactions (Kostenuik & Shalhoub, 2001). OPG is secreted by cells of mesenchymal origin, both basally and in response to other regulatory signals, including cytokines and bone-targeting steroids (Raisz, 2005). Interestingly, pro-inflammatory cytokines (TNF-α, IL-1, PTH) suppress OPG expression while simultaneously enhancing RANKL expression (Schett, 2011).

TNF exerts its effect on osteoclastogenesis by acting directly on osteoclast precursors, as well as indirectly, by upregulating the production of M-CSF and RANKL on mesenchymal cells (Abu-Amer et al., 2000; Lam et al., 2000). Although there remains debate regarding whether TNF can induce osteoclastogenesis independently of RANKL, the results of studies in animal models of inflammatory arthritis in which RANK signaling is abrogated, indicate that RANKL is required for TNF-induced osteoclast formation (Pettit et al., 2001; Redlich et al., 2002). TNF assails bone by simultaneously inhibiting the expected homeostatic response of new bone formation. Furthermore, it also impairs the function of bone-forming osteoblasts in three ways: (1) by suppressing mature osteoblast function such as the production of a matrix that is competent for mineralization, (2) by blocking the differentiation of new osteoblasts from their progenitors, and (3) by inducing osteoblast resistance to vitamin D, 1,25 dihidroxyvitamin D (1,25 $(OH)_2D_3$) (Hill et al., 1997; Hock et al., 2001; Jilka et al., 1998; Kitajima et al., 1996). IL-6 is produced by osteoblasts, monocytes and T-cells and has been implicated in the pathogenesis of various metabolic bone diseases, including postmenopausal osteoporosis (Jilka et al., 1992). IL-6 can upregulate RANKL and thus indirectly support osteoclast formation via the interaction with mesenchymal cells (Udagawa et al., 1995).

Both type I IFNs and IFN-γ suppress osteoclast differentiation (Takayanagi et al., 2000). IFN-γ is the leading cytokine expressed by TH1 lymphocytes TH1 cells and has important functions in host defense (Quinn et al., 2008). The suppressive function of IFN-γ on osteoclast differentiation is based on interference with RANKL signalling, where IFN-γ constitutes a negative feedback loop for RANKL-mediated osteoclast activation (Takayanagi et al., 2000).

Finally, CRP, a member of the pentraxin family of innate immune recognition proteins, is regarded as a sensitive marker of systemic inflammation (Black et al., 2004). CRP is predominantly produced in the liver, and IL-1, IL-6 and TNF-α have been identified as regulators of CRP production (Yoshida et al., 2002).

In addition, it should be noted that chronic inflammation is also associated with increased age (Hager et al., 1994) and obesity (Frohlich et al., 2000; Visser et al., 1999), being the preceding condition commonly linked to aging as well. Evidence suggests that exercise has a long-term anti-inflammatory effect which is partly mediated by myokines (mostly the muscle-derived IL-6) and also via a reduction in visceral fat mass (Petersen & Pedersen, 2005). Thus, the independent effects of exercise training on BMD and inflammatory response without weight loss need to be determined.

Nutritional Mechanisms of age-related bone loss

Many nutrients and food components can potentially have a positive or negative impact on bone health (Table 2). They may influence bone by various mechanisms, including alteration of bone structure, the rate of bone metabolism, the endocrine and/or paracrine system, and homeostasis of calcium and possibly of other bone-active minerals (Cashman, 2007). These dietary factors range from inorganic minerals (e.g., calcium, magnesium, phosphorus, sodium, potassium, and various trace elements) and vitamins (vitamins A, D, E, K, C, and certain B vitamins), to macronutrients, such as protein and fatty acids.

Table 2 – Potential nutritional determinants of bone health*. *Based on Chashman (2007)*

BENEFICIAL FACTORS: *Nutrients*	POTENTIALLY DETRIMENTAL DIETARY FACTORS: *Dietary factors/nutrients*
Calcium	Excess alcohol
Copper	Excess caffeine
Zinc	Excess sodium
Fluoride	Excess fluoride
Magnesium	Excess/insufficient protein
Phosphorus	Excess phosphorus
Potassium	Excess/insufficient vitamin A
Vitamin C	
Vitamin D	
Vitamin K	
B vitamins	
n-3 Fatty acid	
Protein	
Phytoestrogens	
Nondigestible oligosaccharides	

* Some nutrients could be categorized as being both beneficial and detrimental depending on dietary exposure level: insufficient or in excess.

We will briefly describe the potential effects of some key nutrients and their known mechanisms of actions by which they play a role in fighting age-related bone loss, and, in opposition, in increasing the incidence of osteoporosis. The importance of calcium and vitamin D in promoting bone health is summarized on the next topic.

It is now established that protein is both detrimental and beneficial to bone health, depending on a variety of factors, including the level of protein in the diet, the protein source, calcium intake, weight loss, and the acid-base balance of the diet (Heaney & Layman, 2008). Protein intake affects bone in several ways: 1) it provides the structural matrix of bone, 2) it optimizes insulin-like growth factor 1 (IGF-1) levels, 3) it is reported to increase urinary calcium, and 4) it is reported to increase intestinal calcium absorption (Heaney & Layman, 2008). Interestingly, findings regarding the effect of protein on calcium balance and bone health have been mixed (Hunt et al., 2009; Kerstetter et al., 2005; Kerstetter et al., 1998). Increased calciuria does not necessarily translate to calcium loss, negative calcium balance, and reduced bone mass (Bonjour, 2005). Overall, however, there is general agreement that moderate protein content diets (≈1.0 to 1.5 g/kg/ day) are associated with normal calcium metabolism and do not alter bone metabolism (Kerstetter et al., 2003).

Although a depletion of phosphorus leads to impaired mineralization, there is more concern on the effects of high dietary phosphorus on bone, especially if combined with

a low calcium diet (Palacios, 2006). High phosphate levels in the blood reduce the formation of 1,25 $(OH)_2D_3$ in the kidneys, reduce blood calcium, and lead to increased PTH release by the parathyroid glands (Ilich & Kerstetter, 2000).

Despite the generalized analysis of the effect of each isolated nutrient on bone health, attention should also be placed on the combined effect of multiple nutrients. For example, bone loss may be attributable, in part, to the mobilization of skeletal salts to balance the endogenous acid generated from acid-forming foods (Krieger et al., 2004). By preserving calcium in bones, which might otherwise be mobilized to maintain normal pH, potassium rich foods may help to prevent osteoporosis (Ilich & Kerstetter, 2000).

This relationship may explain the reported beneficial influence of fruit and vegetables on bone health (New, 2003; New et al., 2000). The detrimental effect of dietary acidity on the skeleton is relatively small (Welch et al., 2007), but a sustained small effect may have a large impact over time (New, 2003).

Protein and cereal grains are metabolized to acidic residues whereas fruits and vegetables have an alkaline residue (containing the cations potassium, calcium, and magnesium). Therefore the balance between intakes of these major dietary components will determine the net potential acid load of a diet (Remer & Manz, 1995). An excess acid load is buffered by bone and in the process calcium is released. Epidemiologic studies have observed that greater intakes of fruits and vegetables are associated with greater BMD (Chen et al., 2006; New et al., 2000), and acidic environment leads to progressive bone loss (Macdonald et al., 2005; Tucker et al., 2001). Algorithms based on dietary intakes of key nutrients can be used to measure of acid-base load, such as the dietary potential renal acid load (PRAL), and to explore the association between dietary acidity and bone health (Macdonald et al., 2005).

It is suggested that metabolic acidosis stimulates physicochemical mineral dissolution and subsequently cell-mediated bone resorption. Acidosis suppresses the activity of bone-forming cells, osteoblasts, decreasing gene expression of specific matrix proteins and alkaline phosphatase activity (Cao & Nielsen, 2010). There is a concomitant acid stimulation of prostaglandin production by osteoblasts, which acting in a paracrine manner increases synthesis of the osteoblastic RANKL. The acid induction of RANKL then stimulates osteoclastic activity and recruitment of new osteoclasts to promote bone resorption and buffering of the proton load (Krieger et al., 2003). Both the regulation of RANKL and acid-induced calcium efflux from bone are mediated by prostaglandins (Krieger et al., 2004).

Several other micronutrients with essential roles in bone health may also be inadequate in the diets of the elderly. They are vitamin K, vitamin C, vitamin A, magnesium, boron, and other trace minerals. Less research has been conducted on these micronutrients compared to calcium, phosphorus, and vitamin D, but they nevertheless are essential for bone health. Indeed, magnesium plays an important role in calcium and bone metabolism. It has direct effects on bone quality by decreasing hydroxyapatite crystal size, thereby preventing larger, more perfect mineral crystals that result in brittle bone (Palacios, 2006). Deficiency of this mineral could affect bone growth, osteoblastic and osteoclastic activity, osteopenia, bone fragility, and alter calcium metabolism (Rude et al., 2009) resulting in hypocalcemia, vitamin D abnormalities and neuromuscular hyperexcitability.

Finally, the negative influence of caffeine on bone health has been supported in previous studies (Harris & Dawson-Hughes, 1994; Massey & Whiting, 1993) but not consistently (Heaney, 2002). Caffeine is negatively correlated with intestinal calcium absorption with the net result being a more negative calcium balance, which is most pronounced when dietary calcium is inadequate (Harris & Dawson-Hughes, 1994; Massey & Whiting, 1993; Rapuri et al., 2001).

Calcium and vitamin D and the aging skeleton: efficacy in the treatment of osteoporosis

Physiological alterations associated with aging process may make the elderly population most susceptible to vitamin D deficiency and its consequences. Age-induced skin changes reduce the amount of 7-dehydrocholesterol, the precursor of cholecalciferol (vitamin D3), as well as its rate of conversion (Tuohimaa, 2009). Absorption of dietary vitamin D is also reduced in older individuals (Christakos et al., 2011). The aging adult also has a reduction in the quantity and activity of the renal 1-alfa-hydroxylase, which affects the production of the most active metabolite of vitamin D, 1,25 $(OH)_2D_3$ (Tuohimaa, 2009).

The active form of vitamin D plays a vital role in promoting intestinal calcium absorption, being this one of the most important functions attributed to vitamin D in the development and maintenance of skeleton mass. In addition, advanced age is associated with diminished renal and hepatic conversion of vitamin D precursors,

decreased renal response to PTH, and increased resistance of intestinal mucosal cells to the active form of vitamin D (Lanham-New, 2008).

Physiological changes in vitamin D metabolism are not the only changes that affect the vitamin D status in older individuals. Sunlight deprivation for a variety of reasons, the increased use of medications that may interfere with vitamin D metabolism, and the greater likelihood of medical conditions (e.g., renal disease, severe hepatic disease, and malabsorption) that can also interfere with vitamin D metabolism all contribute to a greater prevalence of vitamin D deficiency in older adults (Ilich & Kerstetter, 2000; Tuohimaa, 2009).

However, extensive clinical and animal studies support the concept that vitamin D deficiency and altered vitamin D metabolism contribute to bone loss, hip fractures, osteomalacia, and reduced muscular function (Lips & van Schoor, 2011), which may increase risk of falling.

There is extensive evidence that 1,25 $(OH)_2D_3$ stimulates both bone formation and resorption, however the mechanisms of such actions remain unclear. As osteoblastic cells contain the vitamin D receptor (VDR), the actions of 1,25 $(OH)_2D_3$ on bone formation likely result from a direct stimulatory action of the steroid hormone on the osteoblast differentiation and osteoblastic synthetic functions (Song et al., 2011). Moreover, 1,25 $(OH)_2D_3$ also stimulates calcium transport across the intestinal cells by inducing the production of a calcium-binding protein. Hence, vitamin D is critical for effective calcium absorption (Christakos et al., 2011), but is less important at high calcium loads.

Despite these observations, vitamin D supplementation alone does not appear to reduce the incidence of hip or vertebral fractures, but the use of vitamin D in combination with calcium has been shown to be effective in reducing the risk of vertebral and nonvertebral fractures, including hip fractures (Avenell et al., 2009).

Frequently, older adults are unable to obtain adequate amounts of calcium from the diet (Gennari, 2001). Thus, to smooth out fluctuations in calcium concentration, bone resorption is increased and bone reformation at the same site is also increased to regenerate the calcium supply when the dietary intake of calcium increases (Cashman, 2002). In aging the ability of the kidney and bowel to maintain extracellular calcium homeostasis declines and the reasons are complex (Peacock, 2010). The consequences of these defects are that skeletal bone resorption rises and together

with the deficient bone regeneration associated with aging, the outcome is osteoporosis (Hwang & Putney, 2011).

The flow of calcium into and out of the bone, gut, and kidney is regulated by a variety of mechanisms, which are only partly understood but involve the principal hormonal regulator of calcium homeostasis, the vitamin D-PTH system (Peacock, 2010).

Critical abnormalities in calcium homeostasis resulting in age-related osteoporosis involve all three main organs of calcium homeostasis, summarized in Figure 2.

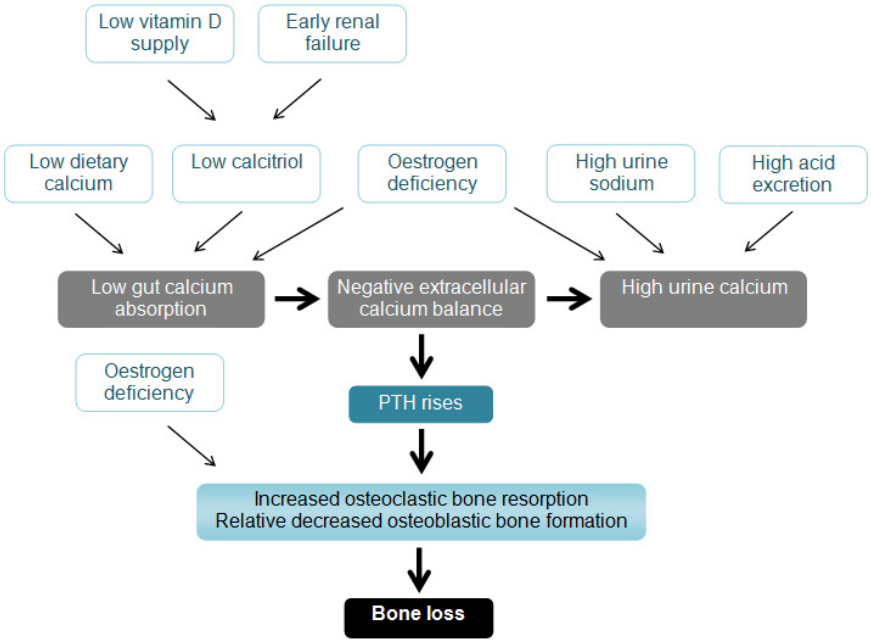


Figure 2 – Schematic model of the disordered mechanisms of calcium homeostasis linked to age-related bone loss

On the supply side there is a reduction in gut calcium absorption (Devine et al., 1993; Morris et al., 1991; Need et al., 1998), involving intrinsic gut wall defects and abnormalities in vitamin D and estrogen status. On the demand side there is an increase in renal calcium excretion that, in women, occurs at menopause and persists during old age (Cashman, 2002) due to estrogen deficiency and other determinants of renal calcium excretion such as salt and acid base balance.

As calcium serves as an indirect regulator of skeletal remodeling, a large number of epidemiologic studies have examined the effect of dietary calcium and calcium supplements on fracture rates and BMD. Most of them found that calcium supplementation increases BMD and is linked to decreased risk for vertebral and hip fractures (Jackson et al., 2006; Jackson et al., 2011; Shea et al., 2004). Accordingly,

calcium seems to function as an antiresorptive agent. It does not antagonize PTH action on bone as do estrogen, the SERMs, and the bisphosphonates, but reduces remodeling by directly reducing PTH secretion (Heaney & Weaver, 2005).

Taken together, these findings demonstrate that calcium and vitamin D are necessary for normal skeletal homeostasis and are considered the first step in osteoporosis treatment.

Genetic lactase nonpersistence (C/T-13910 polymorphism) and bone health

The ability to digest lactose in adults is an autosomal dominant hereditary condition caused by the persistence of lactase activity in the small intestine after weaning. In subjects with lactose malabsorption, the downregulation of lactase enzyme activity occurs after weaning in the intestinal cells to 5–10% of that in infancy (Coelho et al., 2005). Interestingly, lactase non-persistence is the norm rather than the exception, as approximately 70% of the world's population loses the ability to digest large amounts of lactose after weaning (Jackson & Savaiano, 2001). Previous studies have identified that in adulthood, 11–32% of lactose-intolerant subjects report no symptoms from lactose-containing milk products; however, their calcium absorption is reduced (Carroccio et al., 1998; de Vrese et al., 2001).

Enattah et al. (2002) have identified a single nucleotide polymorphism (SNP), a C to T change residing 13910 base pairs upstream of the lactase gene at chromosome 2q21-22, which shows complete association with lactase persistence, with the C/C–13910 genotype defining lactose malabsorption and the genotypes C/T–13910 and T/T–13910 lactase persistence.

The effect of the lactase 13910 C/T polymorphism on BMD have been studied, but finding are not consistent. Obermayer-Pietsch et al. (2004) reported an inverse relationship between this polymorphism and axial BMD in postmenopausal women. Others have failed to confirm this relationship (Gugatschka et al., 2007). Furthermore, lactose intolerance resulted in higher fracture rate on weight-bearing bones (Honkanen et al., 1997) and altered mineralization with increased both osteoid volume and seam widths (Vigorita et al., 1987). One possible confounding factor may be the common association of lactose 13910 C/T polymorphism with reduced intake of calcium (Bacsi

et al., 2009). Moreover, uncertainty exists about the effect of lactase enzyme activity on the absorption of calcium. Indeed, some studies reported a decreased calcium absorption in lactase-deficient subjects (Sahi, 1994), while others presented opposite results (Tang et al., 2007; Vesa et al., 2000).

Despite the existent controversy, the C/C-13910 genotype associated with primary lactose malabsorption could represent a genetic risk factor for bone fractures for elderly people, but additional research is needed to better clarify this topic.

Osteoporosis and fragility fractures in the elderly

Osteoporosis is the most common skeletal disorder characterized by compromised bone strength, which becomes a serious health threat especially for aging postmenopausal women by predisposing them to an increased risk of fracture ("Osteoporosis prevention, diagnosis, and therapy", 2001). Osteoporotic fractures are associated with substantial morbidity and mortality especially in older women (Johnell & Kanis, 2005).

To standardize values from different bone densitometry tests, results are reported as either a Z-score or a T-score, with both expressed as standard deviation (SD) units. The North American Menopause Society (NAMS) supports the World Health Organization (WHO) and International Society for Clinical Densitometry definitions (Kanis, 1994) of osteoporosis in a postmenopausal woman or a man over age 50 as a BMD T-score less than or equal to -2.5 at the total hip, femoral neck, or lumbar spine (at least two vertebral levels measured in the posterior-anterior projection, not the lateral projection) (see Table 3). In addition to diagnosis through densitometry, osteoporosis can be diagnosed clinically (for example, the presence of a fragility fracture), regardless of the T-score (Kanis, 2002).

Table 3 - BMD-based definitions of bone density

Normal:	T-score above (i.e., better than) or equal to -1.0
Low bone mass (osteopenia):	T-score between -1.0 and -2.5
Osteoporosis:	T-score below (i.e., worse than) or equal to -2.5

From the World Health Organization (Kanis, 1994)

Osteoporosis is categorized as either primary or secondary. Primary osteoporosis is usually due to bone loss that occurs with aging. Secondary osteoporosis is a result of

medications (e.g. glucocorticoids) or diseases (e.g. malabsorption) that adversely affect skeletal health ("Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society", 2010).

The clinical significance of osteoporosis lies in the fractures that occur. Many fracture types are associated with osteoporosis, but the hip, spine, forearm and shoulder are the most common sites of osteoporotic fragility fractures (Cooper et al., 2011). Fractures of the neck and trochanteric regions of the femur are currently one of the most serious health care problems and source of morbidity and mortality for the facing aging populations (Marks et al., 2003). Therefore, there is currently an urgent need to prevent the anticipated rise in hip fracture incidence observed in most countries and especially to investigate the underlying causes of this condition. The probability of sustaining osteoporotic fractures varies markedly in different regions of the world. In Europe, the highest risks (very high risk) of hip fracture are seen in Norway, Iceland, Sweden, Denmark, and United States, whereas coutries like Germany, Switzerland, Finland, Greece, The Netherlands, Hungary, Italy, the UK and Portugal have been described as ‘‘high risk’’ countries defined as having a hip fracture probability that lies between 50% and 75% of the risk that is observed in Sweden (Kanis et al., 2002).

BMD can be used to predict an individual’s risk of an osteoporosis-related fracture and is the most commonly measured attribute compared to other qualities of bone. Qualities of bone other than BMD (including degree of mineralization, hydroxyapatite crystal size, collagen structure, heterogeneity of bone microstructure, connectivity of trabeculae, and microdamage) are difficult or impossible to measure in clinical practice at this time. Although BMD is an important component of assessing fracture risk, other factors should also be considered. These include prior fracture, age, family history of osteoporotic fracture, or long-term glucocorticoid therapy, among others (Table 4), all of which should be taken into account in the assessment of fracture risk in patients (Kanis et al., 2005; Miller, 2006).

Despite the contribution of a variety of risk factors, BMD is consistently identified as an important determinant of fracture risk, especially in women age 65 and older (Johnell et al., 2005; Kanis et al., 2005). BMD and fracture risk are most closely related when BMD is used to predict the fracture risk at that same site. Risks for spine fracture and hip fracture increase 2.3-fold and 2.6-fold, respectively, for each decrease of 1 SD in age adjusted BMD (Cummings et al., 1993). Nevertheless, people with low BMD will not always develop fractures, but the probability of fractures is increased. There is a continuous relationship between fracture probability and BMD, i.e., fracture probably

increases progressively as bone density declines. Despite the obvious relationship between these two variables, only 15% of fractures occur in persons with osteoporosis (Siris et al., 2001). In addition, risk factors for hip fracture are more strongly related to predictors of falls and factors that may modify the impact force of a fall (Table 4) than to low bone mass *per se* (Marks et al., 2003).

Table 4 – Risk factors for osteoporotic fractures. Based on ("Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society", 2010; Wilkins & Birge, 2005)

RISK FACTORS FOR OSTEOPOROTIC FRACTURE	FACTORS CONTRIBUTING TO THE RISK OF HIP FRACTURE
Fall	Environmental hazards
Age (50 to 90 years)	Lifestyle habits
Sex	Location of the fall impact
Weight*	Mode of falling
Height*	Property of the fall surface
Low femoral neck BMD	Geometry of the hip
Prior fragility fracture	Distance to impact
Parental history of hip fracture	Degree of soft tissue coverage over the hip bone
Inactivity	Increased height
Current tobacco smoking	Lower extremity muscle weakness/atrophy
Long-term use of glucocorticoids	Poor visual acuity
Rheumatoid arthritis	Neurodegenerative disorders of the central nervous system
Other causes of secondary osteoporosis	Postural instability
Alcohol intake of more than two units daily	Medications affecting postural stability

* Body mass index is automatically computed from height and weight.

As illustrated in table 4, history of fragility fracture is a relevant risk factor for osteoporotic fractures. In fact, after the 40th birthday, history of fragility fracture is associated with a 1.5- to 9.5-fold increased risk of future fracture, depending on the patient's age and the number and site of prior fractures (Klotzbuecher et al., 2000). Also, as inactivity can lead to muscle weakness and atrophy, it is associated with an increased risk of fracture in elderly people (Marks et al., 2003).

Risk factors for falling: The role of exercise in fall prevention

Older adults represent a large and increasing percentage of the population, and aging is associated with an increased risk of falling and consequent injuries. Thus, falls are a common and often devastating problem among older people, engendering

considerable mortality, morbidity and suffering, and high social costs due to premature hospital and nursing home admissions (Moore & Ellis, 2008). In addition, a fall may be the first sign of an undetected illness. Falls risk factors are closely related with fracture occurrence, hence there is normally an overlap between risk factors of these two events. The contribution of falls and fall-related risk factors to fracture risk have been discussed previously on "Osteoporosis and fragility fractures", as reduction of fall incidence is important for fracture prevention as well. Approximately 30% of people over 65 fall each year, and for those over 75 years old the rates are higher (Todd & Skelton, 2004). Most of these falls are associated with one or more identifiable risk factors, which can be broadly classified into two categories: intrinsic and extrinsic factors (Table 5). However, there is a complex causal and dynamic interaction between risk factors in both categories and fall occurrence (Todd & Skelton, 2004). Thus, medical assessment of fall risks and provision of appropriate interventions are therefore challenging. Optimal approaches involve interdisciplinary collaboration in assessment and interventions, particularly exercise, attention to co-existing medical conditions and environmental inspection and hazard abatement (Rubenstein, 2006).

Table 5 – Main risk factors for falls amongst older adults. *Based on Marks et al. (2003)*

INTRINSIC RISK FACTORS	EXTRINSIC RISK FACTORS
- History of falls	- Environmental hazards *(poor lighting, slippery floors, uneven surfaces, etc.)*
- Age	- Footwear and clothing
- Female gender	- Inappropriate walking aids or assistive devices
- Living alone	
- Ethnicity	
- Medicines *(e.g., benzodiazepine, psychotropics, class 1a anti-arrhythmic medications, diuretics, and sedatives)*	
- Medical conditions *(e.g., circulatory disease, depression, arthritis, thyroid dysfunction, and diabetes)*	
- Impaired mobility and gait *(e.g., muscle weakness (strength, endurance as muscle power), gait deficit, balance deficit, and difficulty in rising from a chair)*	
- Sedentary behavior	
- Psychological status - fear of falling	
- Nutritional deficiencies	
- Impaired cognition	
- Visual impairments	
- Foot problems	

There is general agreement that exercise occupies a central role in the maintenance of muscle strength, flexibility, gait, balance and reaction time (Carter et al., 2001), and thus, provides a rationale for exercise intervention trials measuring the efficacy of exercise in the prevention of falls in the elderly. Recent systematic reviews and meta-analyses have documented the effectiveness of exercise programmes and other effective approaches (including multidimensional risk factor assessment, and environmental assessment and modification) (Carter et al., 2001; Chang et al., 2004; Costello & Edelstein, 2008; de Kam et al., 2009; Gillespie et al., 2009; Sherrington et al., 2008). Exercise appears to be a useful tool in fall prevention in older adults, significantly reducing the incidence of falls compared with control groups. Furthermore, greater relative effects are seen in (i) programs that include balance and strength training, (ii) use a higher dose of exercise, and (iii) do not include a walking program.

In summary, exercise can be used as a stand-alone intervention for falls prevention or as a component of a multifaceted program, which seem to have the strongest effects (and cost-effective).

Adaptive bone response to mechanical loading

Evidence from both animal and human studies indicates that exercise in general, and mechanical signals in particular, are both anabolic and anti-catabolic to bone tissue and benefit both bone quantity and quality (Rubin et al., 2008). The general phenomenon of bone responses to mechanical loading comprises two major questions: 1) how bones perceive forces (mechanotransduction), and 2) how do applied loads induce osteogenic response.

Loading derives from forces applied to a bone either from a muscle pulling on an origin or insertion region, or from external forces acting on a bone across a joint or from the outside world (e.g., the ground) (Pearson & Lieberman, 2004). Loads applied to the skeleton are generally described in terms of stress (defined as force per unit area) and strain (Turner & Robling, 2005). Strain is a measure of bone deformation in response to the application of stress and can be calculated by dividing the change bone length by its original values. One strain unit equals a 0.1% deformation (Zernicke et al., 2006). Simply put, force generates stresses of varying intensity, which produce strains of varying magnitude and mode.

The precise strain level actually “experienced” by bone cells *in vivo* is not known, and may be as much as ten times that experienced by the matrix (Nicolella et al., 2006). Bone cells also experience interstitial fluid flow through the canaliculi surrounding the osteocytes inducing shear stress and deformation of the cell membrane (Bonewald, 2006). Functional loading also induces pressure in the intramedullary cavity, shear forces through canaliculi, and dynamic electric fields as interstitial fluid flows past charged bone crystals. Certainly, the complex loading environment of the skeleton generates a diverse range of mechanical signals that are ultimately inseparable, but may differentially influence tissue, cell, and molecular activity (Rubin et al., 2008).

Once a bone experiences some type of strain that is sufficient to be sensed and transduced, the next step in any bone response to mechanical loading is the activation of osteogenic cells to elicit a potential outcome (Pearson & Lieberman, 2004). There are several mechanical factors regulating bone cell response, thus not all loading regimens have equal osteoregulatory effects. Studies with animals show that bone remodeling is sensitive to changes in strain magnitude (Frost, 1993; Rubin & Lanyon, 1985), the number of loading cycles (Hsieh et al., 2001; Rubin & McLeod, 1994), the distribution of the loading (Lanyon et al., 1982), and the rate of strain (O'Connor et al., 1982). Importantly, the load signal must be dynamic (time-varying) as static loads are ignored by the skeleton (Lanyon & Rubin, 1984), whereas the anabolic potential increases when rest periods (recovery period to reestablish their mechanical sensitivity before they can fully respond again to their mechanical environment) are inserted between the mechanical events (Srinivasan et al., 2002).

The sensitivity of bone cells to mechanical signals, including stromal cells, osteoblasts, and osteocytes, has been well documented (Rubin et al., 2006) but it is difficult to designate a critically responsive cell. Whereas the osteoblast is critical for the adaptive response, the osteocytes may have a key role in bone tissue plasticity (Rubin et al., 2008). Marrow stromal cells change proliferation and gene expression in response to mechanical stimulation (Li et al., 2004) and through mechanical regulation of RANKL expression (Rubin et al., 1999) also affect osteoclast number and function. The osteoclast also responds directly to mechanical signals limiting bone resorption (Duncan et al., 1992).

Transducing mechanical signals into cellular response require an exquisitely sensate receptive system. Multiple candidates for mechanoreceptors exist: ion channel activity in osteoblasts; membrane spanning integrins which couple the cell to its extracellular environment; and a large number of adhesion-associated linker proteins (Rubin et al.,

2008). With the multiplicity of mechanical signals presented to the cell, it is likely that no single mechanosensor or receptor mechanism perceives the mechanical environment. At the very least, multiple mechanosensors interact to integrate both mechanical and chemical information from the microenvironment. Regardless the molecular mechanisms by which cells (mostly in the osteoblast-lineage) regulate osteoblasts and osteoclasts, the potential outcomes of the above pathways are either quiescence, modeling (periosteal and/or endosteal deposition), resorption, or Haversian remodeling (bone turnover) trought the BMU (Pearson & Lieberman, 2004). In addition, the range of results and the complex mechanical milieu generated by exercise suggests that some components of the load-bearing regimen are more influential than others.

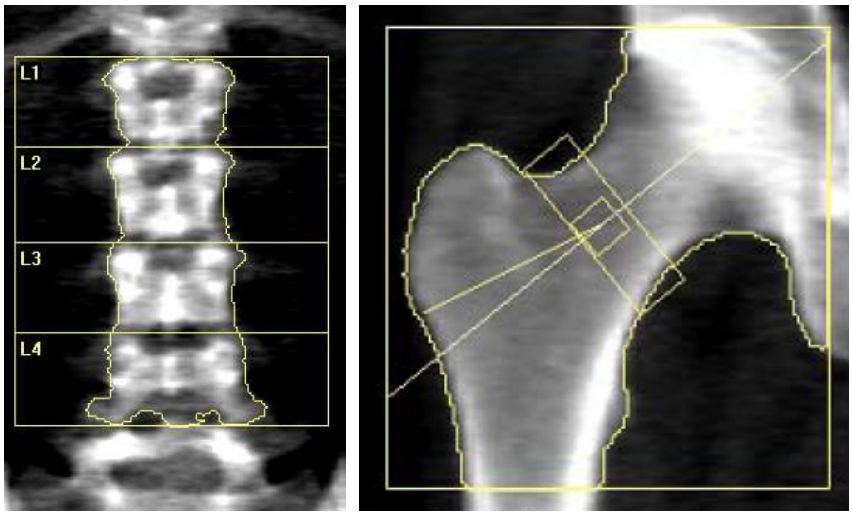
Finally, all of the previously discussed declines in osteoblastic function and sensitivity to hormonal and other chemical signals (see also “Cellular mechanisms of age-related bone loss” pp. 33) combine to make the aging skeleton much less able to build new bone in response to exercise (Khosla & Riggs, 2005; Robey & Bianco, 1999).

Measuring bone properties

Different methods to assess bone mass and architecture have been developed and are normally used for diverse purposes (screening and/or diagnosis of osteoporosis, monitoring preventive and/or treatment efficacy, prediction of future rate of bone loss and prediction of fractures) and for targeting specific bone-related end-points. Methods currently available include dual energy x-ray absorptiometry (DXA), computed tomography (CT), quantitative ultrasound (QUS), magnetic resonance imaging (MRI), and radiography.

All methods have unique features with some potential advantages and disadvantages which may compromise the ability to provide insights into bone properties. DXA, CT and radiography are all X-ray based techniques. Although osteoporosis can often be diagnosed by looking at simple radiographs, this method has low sensibility and is not recommended for diagnosis (Kanis, 2002). On the other hand, radiography may be useful to detect several characteristic features of osteoporosis, including subclinical vertebral fractures (which is a strong risk factor for subsequent fractures) and vertebral deformities due to osteoporosis (Kanis, 2002).

Introduced in the late 1980s, DXA is now the most widely used and available bone densitometry technique (Blake & Fogelman, 2007), and central DXA measures of lumbar spine, femoral neck, and total hip are currently used as the "gold standard" for the clinical diagnosis of osteoporosis by bone densitometry (Figure 3) (Kanis et al., 2008).



A B

Figure 3 - (A) DXA of normal lumbar spine L1-L4.(B) DXA of right hip; although BMD is provided in a number of different sites (femoral neck, oblong box; Ward's area, small box; trochanter; and total hip), for clinical diagnosis of osteoporosis, femoral neck and total hip are used.

Generally, DXA measures the relative tissue absorption of x-rays, with attenuation values being converted to hydroxyapatite-equivalent density (Griffith & Genant, 2008). The most relevant distinctive advantages of this technique include: a fan-bean X-ray source and a bank of detectors that enable faster scanning (1min/site) with improved image quality and spatial resolution (0.5mm), very low radiation doses (spine 2-4 μSv; femur, 2-5 μSv; whole body, 1-3 μSv) that are similar to those of natural background radiation (2400 μSv/yr; ≈7 μSv/d) (Blake et al., 2006), high precision and accuracy (Engelke & Gluer, 2006), assess the specific sites most vulnerable to fracture (Fogelman & Blake, 2005), peripheral DXA units are also available for densitometry of the forearm or calcaneum, hip structural analysis software provides added useful information (Prevrhal et al., 2008), including hip axis length, femoral neck cross-sectional area and moment of inertia, and femoral neck shaft angle, over BMD measurement. Conversely, some technical limitations have consistently been pointed out including: (i) the inability of DXA to measure true volumetric BMD but rather areal density (measured in grams/cm^2), (ii) the strong influence of bone size (since the

relation between area and volume is non-linear), (iii) DXA does not distinguish between cortical and trabecular bone (Griffith & Genant, 2008), and (iv) lack to predict femoral strength and fracture risk based on the spatial distribution of bone mass intrinsic to structural geometric properties, such as cortical thickness, cross-sectional size, length of neck and shaft, and angle of the femoral neck (Faulkner et al., 2006).

The CT methods used to assess bone mass and architecture include standard quantitative CT (QCT), volumetric QCT (vQCT), peripheral QCT (pQCT), high-resolution pQCT and micro-CT. Measurements are reported as true volumetric density (mg/cm^3). The renewed interest in QCT has two primary reasons: the deficiencies of DXA in monitoring treatment antiresorptive drugs (Black et al., 2003) and the prospect that QCT may not only do better but also yield direct measures of strength (and, addressing the combination of these two aspects, variations in strength induced by treatment). Although QCT is less affected by size, it is more sensitive to bone marrow changes (Boutroy et al., 2005) and pQCT results should not be used for diagnosis. The radiation exposure is higher, precision errors of spinal CT approaches are somewhat higher, and spinal QCT have a poor performance in predicting hip fracture than DXA (Gluer, 2008). vQCT can thus analyse both densitometry and geometrical components either of the entire bone or its cortical and trabecular components separately (Griffith & Genant, 2008). Moreover, finite element analysis (FEA) modeling is an established engineering method that can be applied to improve QCT estimation of bone strength (Keaveny et al., 2007). FEA of models based on three-dimensional pQCT images (isotropic voxel resolution of 165 mm) predict distal radial bone strength more accurately than DXA results or bone structural parameters (Pistoia et al., 2002). Micro-CT has a superior resolution but is currently limited to studying biopsy samples and small animals, as the bone size is too small for human use and the radiation exposure required to support the very high resolution would be prohibitive in humans. Some other disadvantages of CT are difficulties with quality control, and the high cost (less accessible) compared with DXA (Griffith & Genant, 2008).

MRI is a nonionizing technique, based on the interaction between a high-gradient magnetic field, radiofrequency pulse transmission, and protons in the tissues under investigation (Griffith & Genant, 2008), which makes possible to obtain noninvasive bone biopsies at multiple anatomic sites. The three competing factors to be considered in high-resolution MRI (HR-MRI) are signal-to-noise ratio (SNR), spatial resolution, and imaging time. Until recently, *in vivo* MRI of trabecular microarchitecture was limited to peripheral sites such as the distal tibia and femur, radius, and calcaneus because of

SNR limitations (Gluer, 2008). However, the main sites of osteoporotic fractures are nonperipheral regions such as the vertebral bodies (spine) and the proximal femur (hip). *In vivo* MRI of trabecular microarchitecture is commonly applied to peripheral sites, and only recently high-resolution has been applied to the proximal femur (Krug et al., 2005) by using SNR efficient sequences, high magnetic field strength (3T), and phased array coils. Several aspects of MRI provide great potential for further investigation of osteoporosis. MR does not involve ionizing radiation, allows direct multiplanar acquisition, and can explore aspects of bone physiology not amenable to investigation by other imaging techniques. MR has been applied to the study of bone architecture, marrow fat content, marrow diffusion, and marrow perfusion (Griffith & Genant, 2008).

QUS measures the attenuation (known as broadband attenuation (BUA), measured in dB/MHz) and speed of an ultrasound wave (speed of sound (SOS), measured in m/s (Damilakis et al., 2007), which may reflect elements of bone quality as well as bone density. Because it is a totally different nonradiographic-based technique, the agreement between DXA and QUS regarding BMD assessment is only modest. In addition, several other parameters of quantitative ultrasound can be measured, such as stiffness, amplitude-dependent speed of sound, bone transmission time, fast wave amplitude, signal dynamic and ultrasound bone profile index (Gonnelli et al., 2005). QUS is relatively portable, easy to use, inexpensive and safe. QUS is currently limited by the reliance on bone density to diagnose osteoporosis, the inherent variability among QUS devices, the higher time period to follow individual subjects compared to DXA (two to three times more for a given rate of change (Damilakis et al., 2007)), and the lack of consensus as to what QUS variables are most predictive of reduced bone strength. Therefore, QUS is primarily used to screen subjects likely to benefit from DXA examination.

Biochemical markers of bone turnover in osteoporosis

Bone metabolism is characterized by two opposite activities coupled at a BMU (Jilka, 2003). During bone resorption, dissolution of bone mineral and catabolism of bone matrix by osteoclasts results in the formation of resorptive cavity and the release of bone matrix components. Then, during bone formation, osteoblasts synthesize bone matrix that fills in the resorption cavity and undergoes mineralization (Szulc & Delmas,

2008). There are two groups of biochemical bone turnover markers (BTMs), and several blood and urinary molecules have been identified as markers for both groups (Table 6), providing estimations of the rates and direction of the biological activities governing bone turnover ("Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society", 2010).

Collection of serum should be performed in standardized conditions, preferably in the fasting state in the morning. Circadian rhythm has a strong impact on the variability of BTMs, especially bone resorption markers that peak in the second half of the night and have their nadir in the afternoon (Bjarnason et al., 2002; Qvist et al., 2002)

Table 6 - Bone turnover markers

BONE FORMATION MARKERS	BONE RESORPTION MARKERS
Serum	*Serum*
Procollagen I N terminal extension propeptide (PINP)	C terminal telopeptide (ICTP)
Bone specific alkaline phosphatase (B-ALP)	Tartrate resistent acid phosphatase (TRACP)
Osteocalcin (OC)	
Procollagen I C terminal extension propeptide (PICP)	*Urine*
	N-terminal type I collagen telopeptide* (NTX)
	C-terminal type I collagen telopeptide* (CTX)
	Deoxypyridinoline (DPD) free or total Hydroxyproline

*Also measurable in serum

During the remodeling process, osteoblasts synthesize a number of cytokines, peptides and growth factors that are released into the circulation. Their concentration may thus reflect the rate of bone formation (Brown & Josse, 2002).

Most bone resorption markers are degradation products of bone type I collagen, except the osteoclast-specific enzyme TRACP 5b, which is secreted by the osteoclast. TRACP activity is thought to reflect osteoclast number and activity rather than the rate of collagen breakdown and can be measured by immunoassay (Seibel, 2005). Other important resorption markers still under investigation are the osteoclast-derived enzymes cathepsin K and L and the non-collagenous bone sialoproteins (Vasikaran, 2008).

BTMs may show an individual response to therapy earlier than BMD changes (Civitelli et al., 2009; Garnero, 2008; Marcus et al., 1999) but they reflect the number of remodeling sites and the current rate of bone remodeling in the whole skeleton, and cannot be used to diagnose osteoporosis (Garnero, 2008).

Nevertheless, and despite some conflicting results, BTMs are of value in estimating bone turnover rates (Garnero, 2008). These tests may be used to identify fast bone losers (Lofman et al., 2005), and have been shown in long-term prospective studies to have some utility in fracture risk prediction (Garnero et al., 1998; Weisman & Matkovic, 2005). In the elderly population BTMs show a strong negative correlation with BMD (Garnero et al., 1998; Zhou et al., 2011). Thus, biochemical indices of skeletal metabolism may have a potential clinical application in fracture risk assessment. Using a combined approach, with BMD and BTMs, could improve fracture prediction in older adults (Civitelli et al., 2009; Garnero, 2008; Seibel, 2006; Szulc & Delmas, 2008).

[2] EXPERIMENTAL WORK

Experimental work

Figure 4 (next page, see legend below) illustrates the main methodological features of the experimental work, detailing for each paper, the sample size, participants' mean age, outcome variables, measurement techniques and the statistical analyses. The complete description for each section is presented in the corresponding paper at the Methods section.

Figure 4 – Schematic model of the experimental work design (type of studie, final sample, mean age, outcome variables, measurement technique, statistical analyses)

6MWT, 6-min walk test • 30sCST, 30-s chair-stand test • AFM, Appendicular fat mass • ALM, Appendicular lean mass • AP anterior–posterior • BMI, body mass index • BMD, Bone mineral density • CIs, confidence intervals • CON, control group • CT, controlled trial • EA elliptical area • ECLIA, Electrochemiluminescence assay • ELISA, commercial sandwich enzyme-linked immunosorbent assay • ET, exercise-training group • FFM, fat-free mass • FM, Fat mass • ITT, intention-to-treat • KE, knee extension • KF, knee flexion • LM, Lean mass • ML medial–lateral • OLS, one-leg stance • OPG, osteoprotegerin • PA, Physical Activity • PRAL, Potential renal acid load • RANKL, receptor activator of nuclear factor kappa B ligand • RCT, Randomized controlled trial •WC, waist circumference • WMD, weighted mean differences • [a] according to NIH protocol

[1] ET was performed twice a week and was designed to load bones with intermittent and multidirectional compressive forces and to improve physical function

[2] RE included 3 sets of 8 repetitions at 70 to 80% of 1 repetition maximum focused on exercises with attachments to the proximal femur and additional complementary upper body exercises. AE program included dynamic activities involving stepping, skipping, walking, jogging, dancing, aerobics and step choreographies at an intensity that gradually increased from 65% to 85% of the heart rate reserve. Both interventions were conducted 3 times per week for 8 months.

[3] Supervised combined training of resistance training 2 d/wk, for 60 min, and weight-bearing and balance training 1 d/wk for 60 min.

	Paper I	Paper II	Paper III	Paper IV	Paper V
	Cross-sectional study	RCT	RCT	CT	Meta-analysis
Final Sample	100 women	ET, n=30 women CON, n=30 women [1]	RE, n=23 women AE, n=24 women CON, n=24 women [2]	24 women 23 men [3]	1339 women 238 men
Mean age	68.7 years	69.1 years	69.0 years	68.2 years	69.0 years
Outcome variables	Height / weight (BMI) LM FM % body fat Android FM Gynoid FM AFM ALM Femoral neck BMD KE and KF strength Dietary intake PRAL PA Lactase nonpersistence Lifestyle behaviors and clinical status	Height / weight (BMI) WC LM FFM FM Femoral neck, Troch, Inter, Total femur BMD Lumbar spine BMD Walking performance Grip strength Muscle endurance Dynamic and static balance EA AP / ML velocity KE and KF strength Dietary intake PRAL PA Lactase nonpersistence Lifestyle behaviors and clinical status	Height / weight (BMI) WC LM FFM FM Femoral neck, Troch, Inter, Total femur BMD OPG RANKL Dynamic and static balance EA AP / ML velocity KE and KF strength Dietary intake PRAL PA Lactase nonpersistence Lifestyle behaviors and clinical status	Height / weight (BMI) LM FM Femoral neck, Troch, Inter, Total femur BMD Lumbar spine BMD TNFα IFNγ IL-6 OPG RANKL CTX OC Vitamin D hs-CRP Dynamic balance Dietary intake PRAL PA Lifestyle behaviors and clinical status	Femoral neck BMD Lumbar spine BMD
Measurement technique	Stadiometer and balance weighing scales DXA Isokinetic dynamometer 4-day dietary record Actigraph GT1M accelerometer /questionnaire C/T -13910 genotype Questionnaire	Stadiometer and balance weighing scales/ heavy-duty inelastic tape[a] DXA 6MWT Digital hand dynamometer 30sCST 8ft UG OLS Force platform/ MATLAB software Isokinetic dynamometer 4-day dietary record Actigraph GT1M accelerometer C/T -13910 genotype Questionnaire	Stadiometer and balance weighing scales/ heavy-duty inelastic tape[a] DXA Biochemical assays: ELISA 8ft UG OLS Force platform/ MATLAB software Isokinetic dynamometer 4-day dietary record Actigraph GT1M accelerometer C/T -13910 genotype Questionnaire	Stadiometer and balance weighing scales/ heavy-duty inelastic tape[a] DXA Biochemical assays: ELISA/ Luminex/ ECLIA/ Cholestech 8ft UG 4-day dietary record Actigraph GT1M accelerometer Questionnaire	DXA
Statistical analyses	Pearson correlation One-way ANOVA Univariate regression analyses Stepwise linear regression	ITT and per-protocol analyses t-tests/ Wilcoxon test Chi-squared tests/ Fisher's exact test Pearson correlation Two-way repeated-measures ANOVA Bonferroni post hoc tests	ITT analysis t-tests/ Wilcoxon test Chi-squared tests/ Fisher's exact test Pearson correlation Two-way repeated-measures ANOVA Bonferroni post hoc tests	ITT analysis t-tests Pearson correlation Two-way repeated-measures ANOVA Bonferroni post hoc tests Effect size	Random-effects model (WMD) 95% CIs Sensitivity analysis Reporting biases (funnel plot, the regression test, and trim-and-fill) Heterogeneity (Q statistic, T^2 and I^2 methods)

Paper I

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Appendicular Fat Mass is Positively Associated with Femoral Neck Bone Mineral Density in Older Women

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Abstract

Objective: In this study we examined the relationships between body fat accumulation and distribution and bone mineral density (BMD) in older women.

Methods: One hundred healthy white women (mean age 68.7 ± 5.5 years) free of medications known to affect bone were enrolled. Lean mass, fat mass (FM), percent body fat, android FM, gynoid FM, appendicular FM (AFM), appendicular lean mass, and femoral neck BMD were measured by dual-energy X-ray absorptiometry. Dietary intake was assessed by 4-day dietary record and potential renal acid load (PRAL) was also calculated. Performance measures included the knee extension and flexion strength measured on an isokinetic dynamometer. Physical activity (PA) was assessed using accelerometers and questionnaire. Lactase nonpersistence was defined by the C/T -13910 genotype. Sociodemographic information, lifestyle behaviors and clinical status were also examined. Stepwise multiple linear regression analysis showed that AFM was the most significant positive predictor of femoral neck BMD. Results: After adjusting for confounders (age, height, age at menopause, PRAL, PA and knee muscle strength), AFM had strong and independent associations with femoral neck BMD (26.4% of variance).

Conclusions: These data highlight that in elderly women, localization of fat mass is more important for bone mass than obesity per se or lean mass. AFM (subcutaneous adiposity) seems to exhibit an independent protective effect on BMD.

Key Words: Adipose tissue – aging – physical activity – bone – body composition.

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INTRODUCTION

Bone health is a critical issue in quality of life of older adults due to the age-related increased rate of bone loss and increased risk of fall-related bone fractures. Thus, as life expectancy continues to rise, prevention or postponement of age-related decline in bone mass is now of high relevance from a public health point of view.

Bone mineral density (BMD) is influenced by determinants such as genetic factors. These factors primarily include heredity, race, age, and sex.[1] Conversely, environmental factors are also intimately involved in the regulation of bone biology. In fact, numerous risk factors for osteoporosis have been identified including decreased physical activity (PA) and muscle strength.[2] Mechanical loading (strain) can affect cells directly by changing their dimensions, or indirectly through intralacunar pressure, shear stresses, or charged fluid flow.[3] In older adults, decreased muscle strength would reduce the loads on bones that had adapted to stronger young-adult muscles.[4] Among the environmental factors, inadequate nutritional intake[2] may also modulate BMD.

BMD is a complex trait with a close relationship to body mass due to the paramount influence of mechanical loading on bone development and maintenance.[5] Thus, an extensive literature has been developed exploring the linkage between parameters of soft tissue mass, i.e. lean mass (LM) and fat mass (FM), and osteoporosis.[6-8] Since this is a complex issue with many confounding factors, there have been many different conclusions as to the role of the different tissues and bone density. The widely held view that obese women have higher BMD compared with normal weight women, has recently been challenged by conflicting data which suggests that FM is not associated with, nor is negatively related to, bone mass.[8-10] Moreover, epidemiologic studies varied according to whether FM or LM showed the strongest correlation with BMD (reviewed in detail elsewhere[6]). Most studies have consistently focused on total fat accumulation which makes unclear whether a greater phenotypic characterization would account for this relationship. In fact, based on limited research, it is suggested that android fat, apart from increasing the risk of chronic diseases such as cardiovascular disease and type 2 diabetes, is deleterious to bone.[11, 12] Understanding the theoretical divergent or more marked contribution of a specific phenotype is important because body composition is potentially modifiable and interventions promoting healthy body composition in older adults may contribute to maintaining bone health along with cardiovascular condition, and may therefore improve quality of life.

However, a large body of literature on the soft tissue-BMD relationship fails to include nutritional intake and acid-based balance, muscle strength and PA as potential confounding variables,[7, 8, 13] and most of the methods used to assess PA have poor reliability.[14, 15]

Therefore, the main purpose of this study was to examine the relationships between patterns of adipose and lean tissue deposition and BMD in older women, independently of age, age at menopause, height, PA, muscle strength, nutritional intake and acid-base load. We hypothesized that fat mass accumulation would be more closely related to BMD than lean mass.

MATERIALS AND METHODS

Participants and experimental design

Participants were recruited through advertisements in the Porto area newspapers for participation in this university-based study. The eligible participants pool were restricted to older adults with the following characteristics: aged ≥60 years, white, community-dwelling status, not engaged in resistance or water-based exercise training, free of hormone therapy use for at least 2 years, lack of use of any medication and nutritional supplements known to affect bone metabolism (such as diuretics, corticosteroids, anticonvulsants, immunosuppressive medications, non-steroidal anti-inflammatory drugs, asthma medications with corticosteroids,

vitamin D, and calcium), and lack of diagnosed or self-reported cardiovascular, pulmonary, metabolic, renal, hepatic or orthopedic medical conditions.

On the initial screening visit, all participants (113 women, mean age 70.1±6.8) received a complete explanation of the purpose, risks and procedures of the investigation. After signing a written consent form, the past medical history and current medications of the participants were determined. Nine women were excluded due to medical reasons (five used medication known to affect bone metabolism, and four had musculoskeletal disorders that contra-indicate participation in exercise testing) and four were excluded due to current involvement in water-based activities.

Participants' characteristics are listed in Table 1. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human participants were approved by the institutional review board.

Measurements

All measurements were performed by the same evaluator on each test at the faculty facilities on two occasions. On the first assessment, participants completed the lifestyle and clinical status questionnaire, were instructed to use the accelerometer, and DNA samples were collected. The second evaluation took place seven or more days later (to allow seven full days' PA recording and to fill out the 4-day dietary records) and participants completed body composition, bone measurements, and muscle strength tests. All test stations were organized in a circuit, and the same conditions were maintained for each test.

Bone and Body Composition

Dual-energy X-ray absorptiometry (DXA; QDR 4500A; Hologic, Bedford, MA) was used to measure LM, FM, and regional FM in kilograms through whole-body scans while BMD was measured at the femoral neck as described previously.[16] Osteopenia and osteoporosis were classified according to t-score values, and femoral neck fracture threshold (≈0.600 g/cm^2) was also determined.[17] LM was calculated by subtracting the bone mineral content from nonfat mass. Appendicular skeletal lean mass (ALM) and fat mass (AFM) were generated as the sum of LM and FM in arms and legs and were determined by the region of interest (ROI) program. Additional android and gynoid regions were defined using the software provided by the manufacturer (Fig. 1). For the android region, the lower boundary was the top of the pelvis line of demarcation. The upper boundary was placed above the pelvis line of demarcation at a position that was equivalent to 20% of the distance between pelvis and femoral neck. Lateral boundaries were the lines for the arms when in normal position for a whole body scan. The gynoid region was defined with the upper boundary positioned below the pelvis cut line by 1.5 times the height of the android region. The lower boundary was positioned such that it was equal to two times the height of the android region. The lateral boundaries were the outer leg lines of demarcation. Android to gynoid fat ratio was calculated using the FM within the android and gynoid fat regions of interest.

Data collection for other covariates

Height and body mass were recorded using a portable stadiometer and balance weighing scales, respectively. BMI was calculated as body mass (kilograms) divided by height (meters) squared and categorized according to established cut points of <25, 25–30, and ≥30;[18] obesity was considered as BMI ≥30 kg/m^2.

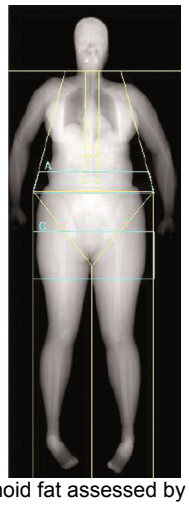

FIG. 1. Regions of trunk, legs, android fat, and gynoid fat assessed by DXA

The dynamic concentric muscle strength of the dominant lower limb, namely knee flexion (KF) and extension (KE) muscle groups, was measured on an isokinetic dynamometer (Biodex System 4 Pro; Biodex, Shirley, NY). Strength measurements were carried out in accordance with the manufacturer's instructions for KE/KF at 60°/s (1.05 rad/s) as described elsewhere.[16] Peak torque, represented as a percentage normalized to body weight, was used for the statistical analyses. The coefficient of variation was 5.1% and 5.5% for KE and KF repeat measurements, respectively (conducted with the same machine by the same examiners).

Historical levels of PA or sport activity were measured by the response to the question "Do you, or did you ever, practice sports or physical exercise sufficient to produce sweating or shortness of breath?" Possible responses were: <1 hour per week, scored low; 1–2 hours per week, scored moderate; and >2 hours per week, scored high.
The Actigraph GT1M accelerometer (Manufacturing Technology, Fort Walton Beach, FL) was used as an objective measure of current daily PA for seven consecutive days using a 15-s measurement interval (epoch) as described previously.[16] One file was corrupt and five files had only three valid days. Those six participants were contacted and agreed to wear the accelerometer again for seven days. In total, final data from all participants was included in the analysis (89 files with 7 valid days, 5 files with 6 valid days, and 6 files with 5 valid days). The average daily minutes of moderate to vigorous PA, number of steps, and daily activity counts per minute was analyzed.

Current nutritional status was assessed using 4-day diet records over three weekdays and one weekend day. To ensure standardization of the dietary records a nutritionist gave individual instruction to the participants concerning how to fill out the diet records and assess food serving sizes. Diet records were analyzed using the Food Processor Plus® (ESHA Research, Salem, OR). Some traditional Portuguese dishes were added to the nutritional data base using Portuguese food composition data. Energy, macronutrients, and other food components that are related to bone metabolism[19] were analyzed, including: protein, calcium, potassium, phosphorus, sodium, magnesium, zinc, vitamin C, vitamin D, and caffeine. Given the increasing evidence that acid-base status has a significant effect on the skeleton, potential renal acid load (PRAL) was calculated by using the algorithm:
PRAL (mEq/d) = 0.49 x protein (g/d) + 0.037 x phosphorus (mg/d) - 0.021 x potassium (mg/d) - 0.026 x magnesium (mg/d) - 0.013 x calcium (mg/d).[20] Nutritional supplements were not considered for total nutritional intake because supplement intake was sporadic and dosages/types for some women were difficult to record.

Lactose persistence mutation C/T -13910 was genotyped by direct sequencing. A 359-bp fragment containing all mentioned mutations and located in intron 13 of the *MCM6* gene was

amplified using primers 5'-GCAGGGCTCAAAGAACAATC-3' (forward) and 5'-TGTTGCATGTTTTTAATCTTTGG-3' (reverse). PCRs contained 0.5μM of each primer, 0.2mM of each deoxynucleotide triphosphate (dNTP), 750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 1.5 mM $MgCl_2$ and 1 U *Taq* polymerase. The PCR profile consisted of 94°C for 5 min, 35 cycles of 94°C for 1 min, 58°C for 1 min and 72 °C for 1 min, followed by 20-min extension at 72°C.
Sequencing reactions were carried out using the ABI Big Dye v3.1 Ready Reaction Kit and using the protocol specified by the manufacturer (Applied Biosystems, Foster City, CA). Products were run on an ABI PRISM 3130x1 sequencer and analyzed in the ABI PRISM 3130x1 Genetic Analyzer software (Applied Biosystems). The resulting chromatograms were inspected for the presence or absence of lactase mutations using the MEGA4.0 software (www.megasoftware.net).[21] DNA was obtained from buccal swabs using standard extraction methods.

A self-administered questionnaire to assess the impact of present and past lifestyle choices was completed by interview, so as to avoid misinterpretation of items and/or skipping of questions. The questionnaire included information regarding educational attainment (use as a marker of socioeconomic status), marital status, fall and fracture history, medical history, current medical conditions, medication use, current and past PA, age at menarche and menopause, current and previous use of hormone-replacement therapy, and current and past smoking habits. Tobacco smoking was recorded in two categories: smokers (current and past, one year or more) and non-smokers. Information on years of education was recorded as the number of years of formal schooling completed by the participants (range = 0 – >12), and was categorized into 3 groups: one to six years, scored low; seven to 12 years, scored medium, more than 12 years, scored high.

Statistical analysis

All statistical analyses were performed using PASW Statistics (version 18; SPSS, Inc., Chicago, IL) for Windows with a significance level of .05 (2-tailed). Data were checked for distribution, and the means ± SD were calculated. Pearson correlations were used to examine associations between covariates and fat mass, lean mass and BMD variables. Potential differences among groups (such as BMI categories) were evaluated using one-way analysis of variance (ANOVA). Univariate regression analyses were used to determine the influence of body composition variables (android fat, gynoid fat, android to gynoid fat ratio, fat mass, percent fat, appendicular fat mass, lean mass, percent lean mass and appendicular lean mass), expressed as continuous variables to femoral neck BMD. Stepwise linear regression was performed to evaluate the strength of the relationship between body fat distribution (including every body composition variables, treated as ''independent'' factors) and femoral neck BMD (treated as outcome variable) to explore what are the important variables influencing the outcome. A predictor was entered into the model at $p \leq .05$ and was removed at $p \geq .10$. Using multiple regression model, covariates such as PA, PRAL, knee muscle strength, age, height, and age at menopause were included because of their association with bone and body composition. Body weight was not included as a controlling variable to avoid multicollinearity, but we included height as a covariate in the model to adjust for the confounding effect of appendicular length. To assess multicollinearity of the regression model, we checked the variance inflation factor. Values above 2 were used to indicate that the model is problematic.

RESULTS

The participants' characteristics are presented in Table 1. The prevalence of obesity was 32% and obese women had significantly higher BMD at femoral neck compared to normal weight women. Of note, the percentage of women with osteopenia and osteoporosis in the femoral neck was 68.5% and 7.9%, respectively, and femoral neck BMD was above the fracture

threshold in only 15 women. On average, participants obtained 81 min of moderate to vigorous PA per day. Past PA habits were predominantly low (55.4%), while only 24.3% had high level of sport participation.

TABLE 1 - General and body composition characteristics of the study population

Variable	113 Women (Mean ± SD)
Age (years)	68.71 ± 5.47
Age at menopause (years)	48.11 ± 5.09
Lactase persistence (%)	55.60
Height (cm)	152.47 ± 6.05
Weight (kg)	65.64 ± 9.97
BMI (kg/m^2)	28.25 ± 4.12
Fat mass (%)	38.55 ± 4.97
Lean mass (%)	58.24 ± 5.49
Android Fat (kg)	2.45 ± 0.92
Gynoid Fat (kg)	4.50 ± 1.16
Android to Gynoid Fat Ratio	0.54 ± 0.13
Appendicular Fat mass (kg)	12.42 ± 2.62
Appendicular Lean mass (kg)	15.44 ± 2.01
Femoral neck BMD (g/cm^2)	0.69 ± 0.10
T- Score	-1.48 ± 0.92
KE/Peak torque BW (%)	138.07 ± 35.20
KF/Peak torque BW (%)	70.74 ± 17.88
Moderate to Vigorous Physical Activity (min/d)	80.07 ± 32.85
Daily counts per min	355.93 ± 118.72
Daily step count	8991.21 ± 4962.65
Nonsmoker (%)	96.7

Proportional distributions are presented for categorical variables. *BMI* Body mass index, *BMD* Bone mineral density, *KE* knee extension, *KF*: knee flexion, *BW* body weight.

The study population had a low educational status, 54.4% completed one to six years of formal schooling, 31.1% completed seven to 12 years, and the remaining 14.5% had more than 12 years of school attendance. Means (±SDs) for nutritional variables are presented in Table 2. Average daily protein intake was 1.01 g/kg body weight and average calcium: protein and calcium: phosphorus ratios were 10.3 and 0.7, respectively.

TABLE 2. – Daily dietary intake at screening.

Energy (kcal/d)	1489.01 ± 347.24
Total fat (% TEI)	30.63 ± 13.26
Carbohydrates (% TEI)	56.69 ± 27.20
Protein (% TEI)	17.83 ± 3.59
Protein (g/d)	65.05 ± 15.51
Calcium (mg/d)	653.86 ± 281.19
Phosphorus (mg/d)	982.83 ± 268.17
Potassium (mg/d)	2461.52 ± 724.66
Sodium (mg/d)	1443.74 ± 446.94
Magnesium (mg/d)	243.45 ± 77.94
Zinc (mg/d)	6.39 ± 6.80
Vitamin C (mg/d)	78.13 ± 53.50
Vitamin D (µg/d)	2.16 ± 1.96
Caffeine (mL/d)	45.07 ± 49.30
PRAL (mEq/d)	-8.70 ± 0.36

Figure 2A shows the univariate linear regression between measures of body fat distribution and femoral neck BMD. All measures were significantly associated with femoral neck BMD, but android to gynoid fat ratio and percent fat mass had the lowest values (adjusted R^2 = 0.059 (p=0.021) and 0.074 (p=0.010), respectively. For lean mass distribution (Fig. 2B), all the relations achieved statistical significance, explaining ≈12% of the variance. As can be noted BMD is positively related to absolute lean mass and ALM, but inversely related to percent lean, since percent lean and fat mass are the converse of one another.

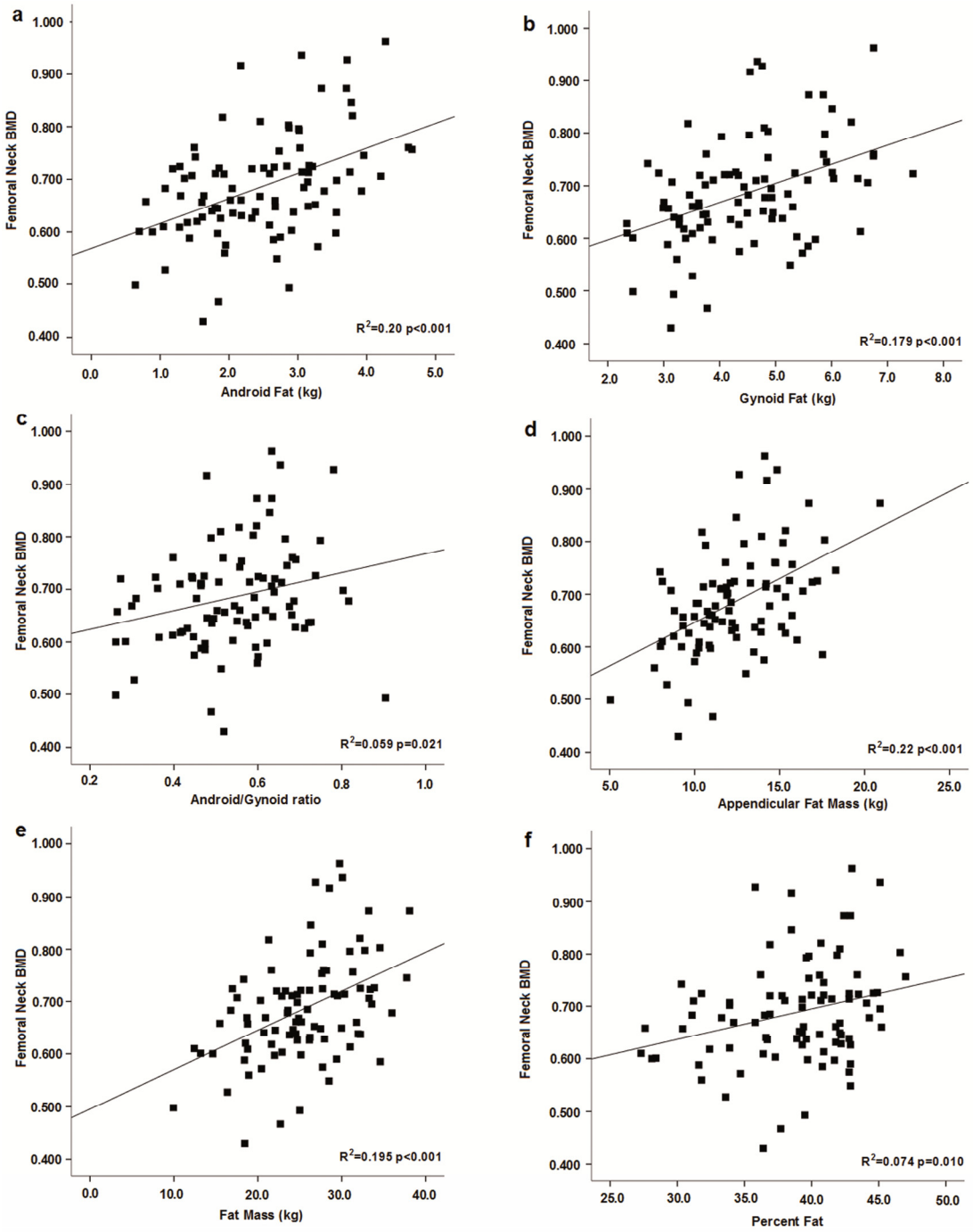

FIG. 2. A: Regression relationships between femoral neck bone mineral density (g/cm2) and fat mass in 100 older women

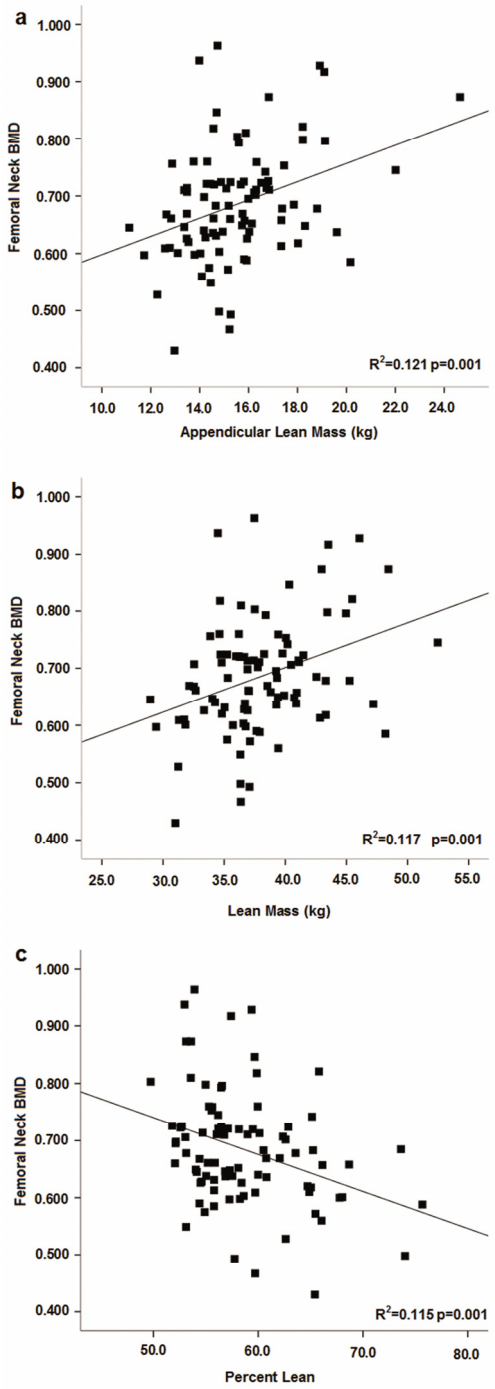

FIG. 2. B: Regression relationships between femoral neck bone mineral density (g/cm2) and lean mass in 100 older women

Educational level and past PA habits were not correlated with BMD, thus they were not included as confounding variables in the regression models. Smoking status was also excluded due to the small prevalence of smokers in the study population (3.3%). Nutritional variables were associated with body composition parameters but not with BMD. Lactase persistence status was not associated with any relevant bone and body composition variable (data not shown). The results of multiple linear regression analyses are shown in Table 3. Stepwise multiple linear regression analysis indicated that AFM had strong and independent associations with femoral neck BMD (R^2=0.169, p<0.001, unadjusted model). Using multiple linear regression and adjusting for all covariates (age, height, age at menopause, PA, knee muscle strength and PRAL), AFM remained statistically significant, accounting for 26.4% of femoral neck BMD variance (Table 3, adjusted model). With every increase of 1 kg in AFM, the femoral neck BMD (on average) increases by 0.023 (95% CI 0.010 to 0.036) g/cm^2, p=0.001.

Covariates such as height, age at menopause, PA, knee muscle strength and PRAL had no significant associations with BMD in regression models; age showed a negative association with BMD (Beta= -0.286, p=0.015; data not shown). AFM was the strongest predictor of BMD (Beta =0.422). No multicollinearity effect was identified by a variance inflating factor between 1.0 and 1.4.

DISCUSSION

The main finding of this work was that AFM, among all included body composition parameters (FM, percent FM, android fat, gynoid fat, android to gynoid fat ratio, ALM, and LM) was the most significant predictor of BMD in older women, which is consistent with our hypothesis.

Moreover, after adjusting for age, height, age at menopause, PRAL, PA and knee muscle strength, the positive association between AFM and femoral neck BMD remained significant. Thus, our final regression model explained 23.4% of the femoral neck BMD variance.

TABLE 3. Multiple linear regression models of femoral neck bone mineral density

Predictor Variable	Adjusted R^2	B coefficient	p	95% CI	Overall p
Unadjusted [a, b]:					
AFM	.169	0.018	.002	0.007 to 0.030	<.001
Adjusted [c]:					
AFM	.264	0.023	.001	0.010 to 0.036	<.001

AFM appendicular fat mass;
[a] Stepwise multiple regression.
[b] The variables excluded were: android to gynoid fat ratio, gynoid fat, fat mass, lean mass, percent fat, appendicular fat mass, android fat.
[c] Multiple regression adjusted for PA, PRAL, Knee flexion muscle strength, age, height, age at menopause.

To the best of our knowledge, this is the first study that focuses on both multiple fat and lean mass variables and multiple potential covariates of BMD, including age, height, age at menopause, PRAL, PA and knee muscle strength.

The relative contributions of LM and FM to the variation in BMD have previously been hypothesized[22] but are highly contentious. Previous studies have pointed towards different directions, suggesting that LM, not FM, is associated with BMD,[8, 9] while other studies have shown that FM, not LM, is an important determinant of BMD.[23, 24] However, few studies have documented the results of lean and fat distribution, whereas the large number of studies looking at relationships between body composition and BMD have assessed only total FM and LM.[10, 23] Although in a previous study from Warming et al.[25] AFM was also found to be correlated to bone mass, their findings suggested that abdominal fat is more important than peripheral fat for endometrial thickness and bone mass in postmenopausal women.[25] In our regression analysis android fat, gynoid fat, and android to gynoid fat ratio were not considered as significant independent predictors of BMD although in the univariate regression analyses they were all positively associated with femoral neck BMD. Despite suggestions that android fat deposition has a negative association with BMD,[7, 8] the relative effect of android fat, gynoid fat and their ratio on BMD is inconsistent. For instance, several studies have revealed that abdominal fat and waist to hip ratio were positively and significantly associated with bone mass,[25, 26] while others found no statistically significant association between fat distribution and BMD in older non-Hispanic Caucasian women.[9] In contrast, Zillikens et al.[7] suggested that after removing the effect of BMI, android fat deposition as measured by the waist-to-hip ratio, waist-to-thigh ratio, and DXA-based android-to-gynoid fat ratio has no association or a negative association with BMD. However, as highlighted in a recent review by Reid,[22] some statistical analyses are confounded by the multicolinearity between the variables studied, when adjustments are made for body weight or BMI. Do to this, those studies have produced misleading results. In addition, the inconsistency of findings may also be related to the use of diverse methods for measuring bone density, the expression of bone mass as BMC or BMD, sample size, environmental and genetic factors included as covariates, part of the skeleton examined, colinearity between FM and LM and/or to differences in the methods of statistical analysis.

Apart from the controversial results regarding the fat-bone connection, the mechanisms accounting for the fat-bone relationship are established and comprise mechanical load that stimulates bone formation, the secretion of bone active hormones from the pancreatic beta cell such as insulin growth factor production, amylin, and preptin, and the secretion of bone active hormones including estrogens, leptin, adiponectin, and interleukin-6 from the adipocyte.[22] Moreover, adipocytes and osteoblasts derive from a common progenitor, the mesenchymal stem cell.[22] Therefore, our results are in line with most of the available data supporting that adipose tissue, particularly appendicular, exerts an independent effect on bone mass.[5]

Expectedly, our data revealed that age is a significant predictor of BMD in older adults which was previously demonstrated by others.[27] In general, aging is associated with declines in many hormones and trophic factors (such as oestrogen, testosterone, vitamin D, growth hormone, insulin-like growth factor-1, cytokines interleukin-1, and interleukin-6), resulting in a reduced amount of bone tissue.

A number of factors did not emerge as significant correlates and/or predictors of BMD. No effect of lactase nonpersistence and several nutrients were detectable, and exercise and knee muscle strength were also unrelated to BMD (data not shown). However, nutritional variables, exercise and muscle strength had significant associations with FM and LM accumulation and distribution. It has been argued that BMD could be affected by lactase nonpersistence mediated by lower calcium intake and impaired calcium absorption[28] which may predispose older adults to bone fracture. Thus, the lack of significant associations was probably due to the similar calcium intake reported in lactase nonpersistent and persistent participants, as in the present study (data not shown).[29] Because entering several nutrients into the same multiple regression analysis yielded invalid results due to the substantial co-linearity between these variables, we only adjust the regression model to the PRAL. There is increasing evidence that acid-base status has a significant effect on the skeleton, and diets with insufficient alkaline-forming foods or a net acid-producing diet may compromise bone health.[30] However, non-mechanical influences could not replace or control the responses to mechanical loading. Otherwise disuse, paralysis or lack of gravitational forces would have no detrimental impact on bone mass. Thus, to fully investigate the relationship between soft tissue and bone mass, it is necessary to control for the mechanical loading effects of habitual PA in the analyses. In a number of experiments with older adults, exercise groups gained more BMD or at least had slower rates of bone loss than their nonexercising contemporaries.[31, 32] The lack of significant association between moderate to vigorous PA and BMD may be explained by the instrument used to measure PA and the specific characteristic of exercise that confers a positive effect on the skeleton. PA is difficult to measure because it is a complex behavior. In fact, the task is even more difficult for bone research field since not all physical activities influence bone in the same way or to the same extent. It has become clear that the mechanical milieu of a given bone is influenced by exercise mode, intensity, duration, and frequency and the effect on bone mass appears to be site specific.[33] Moreover, although osteogenic responsiveness to load bearing is maintained into old age, it clearly declines with maturity. Accelerometer-based measurement of movement is an accepted method for monitoring PA with reasonable reproducibility,[34] and is far more valid than subjective methods including questionnaires or interviews, particularly in older adults.[35] However, some potentially osteogenic-exercise stimulus such as weight-bearing exercise may not be appropriately captured by accelerometers (strain magnitude and distribution). Based on this relevant limitation, we excluded all women that underwent resistance and aqua-based training, as previously mentioned. Nevertheless, similar results, showing that daily PA did not significantly predict BMD in older adults have been reported by others.[36]

Our study has potential limitations deserving comments. Due to the cross-sectional design, causal relationship between BMD and significant predictors cannot be established. Although participants were properly instructed on how to fill out the diet records and assess food serving sizes, the reliability and validity of 4-day diet records depend on nutrient database appropriateness, participants' compliance, and accuracy of the self-reported data (some may reduce intake, or intentionally or inadvertently may not disclose all foods consumed).[37] Although we have used accelerometers to measure PA, activities involving skeletal loading were not separately analyzed. Finally, the results of this study may not be extensive to all older adults due to the small sample size, coupled with the PA, functional and health status of our sample.

CONCLUSIONS

These data suggest a positive and interactive association of BMD with AFM in healthy older women. The fat-bone connection has relevant public health implications for the design of appropriate strategies to combat age-related bone loss since lower body mass is amenable to change and therefore is a potentially preventable risk factor. Moreover, our data complies with previous evidence suggesting that subcutaneous fat (i.e. appendicular fat mass) is more relevant than android fat mass (visceral fat) to bone heath.

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Paper II

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ORIGINAL RESEARCH

Multicomponent Training Program with Weight-Bearing Exercises Elicits Favorable Bone Density, Muscle Strength, and Balance Adaptations in Older Women

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Abstract Physical exercise is advised as a preventive and therapeutic strategy against aging-induced bone weakness. In this study we examined the effects of 8-month multicomponent training with weight-bearing exercises on different risk factors of falling, including muscle strength, balance, agility, and bone mineral density (BMD) in older women. Participants were randomly assigned to either an exercise-training group (ET, $n = 30$) or a control group (CON, $n = 30$). Twenty-seven subjects in the ET group and 22 in the CON group completed the study. Training was performed twice a week and was designed to load bones with intermittent and multidirectional compressive forces and to improve physical function. Outcome measures included lumbar spine and proximal femoral BMD (by dual X-ray absorptiometry), muscle strength, balance, handgrip strength, walking performance, fat mass, and anthropometric data. Potential confounding variables included dietary intake, accelerometer-based physical activity, and molecularly defined lactase nonpersistence. After 8 months, the ET group decreased percent fat mass and improved handgrip strength, postural sway, strength on knee flexion at 180°/s, and BMD at the femoral neck (+2.8%). Both groups decreased waist circumference and improved dynamic balance, chair stand performance, strength on knee extension for the right leg at 180°/s, and knee flexion for both legs at 60°/s. No associations were found between lactase nonpersistence and BMD changes. Data suggest that 8 months of moderate-impact weight-bearing and multicomponent exercises reduces the potential risk factors for falls and related fractures in older women.

Keywords Bone density · Weight-bearing exercise · Age · Dual-energy X-ray absorptiometry · Mechanical loading

The authors have stated that they have no conflict of interest.

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Falls and fall-related injuries occur commonly among older people, and they are strongly related to the age-related deterioration of the sensory systems; loss of muscle mass, strength, and balance; and decrease of bone mineral density (BMD) [1]. From a public-health perspective, prevention of this complex and multifactorial phenomenon is extremely meaningful to the promotion of health in aging, given the impact of bone fractures on the functional independence and quality of life of older people [2]. Increasing evidence has suggested a central role for falls, not osteoporosis, as the strongest single risk factor for a fracture [3]. Accordingly, strategies targeting the prevention of bone fractures in the elderly should focus on reducing the risk of falls and maintaining or improving bone health.

Among the available and most helpful therapeutic recommendations, exercise has been pointed out as an effective, easily accessible, and affordable intervention [4].

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Relevant research suggests that training in balance and strength is the most effective way to minimize the functional decline that acts as an independent predictor of the risk of falls and bone fractures [5]—namely, low BMD, poor muscle strength, and poor postural balance.

Most of the literature published so far has typically included frail participants, with reduced physical function, low bone mass, and osteoporotic fractures [6]. Thus, evidence to support the efficacy of exercise training to maintain or increase physical functioning and bone health in nonfrail older adults is needed.

Although high-impact activities such as jumps and hopping exercises have been successfully used to increase BMD as a preventive intervention to reduce fracture risk [7], older adults are unable to sustain impact-loading regimens without high risk of injury. Moreover, as the loading level of exercise represents an important mediator of bone adaptation, estimating loads based on calculations of the ground reaction forces (GRFs) may provide an additional contribution to specifically characterize the exercises [8]. These useful estimation procedures have rarely been performed in previous investigations.

In previous studies addressing the osteogenic potential of exercise to modulate bone adaptations in older women, lifestyle factors received relatively little attention. Potentially modifiable characteristics such as diet and physical activity (PA) variables, which have been consistently associated with BMD [9], not always have been included in earlier experimental designs. In fact, although the influence of habitual PA in physical functions and bone health has been recognized [10], this relationship has been less studied. Accelerometry allows objective measurement of PA by the use of a motion sensor which records both the number and magnitude of vertical accelerations generated by human movement, and it has been validated in older people [11]. However, only some studies have included PA as a potential confounding variable, using subjective methods to assess it [12, 13].

Moreover, lactase nonpersistence, which has been associated with bone density [14], should also be seen as a potential confounding variable. Indeed, the ability to digest lactose in adults is an autosomal dominant single-gene trait caused by the persistence of lactase activity in the small intestine after weaning [15]. The C/T -13910 polymorphism has been associated with lactose persistence [16].

Therefore, the aim of this study was to assess the effects of an 8-month program of multicomponent training with weight-bearing exercises on different risk factors of falling, such as muscle strength, balance, agility, and BMD, in community-dwelling older women, considering the coinfluence of the referred variables such as diet, PA levels, and lactase persistence and characterizing exercises by providing the GRF as well.

Materials and Methods

Subjects and Experimental Design

Subjects were recruited through advertisements in Porto area newspapers for participation in this university-based study. Seventy-two Caucasian older women volunteered to participate in the study. The eligible subject pool was restricted to older women with the following characteristics: free of hormone therapy use for at least 2 years; aged 60–95 years; community-dwelling status; not engaged in regular exercise training in the last year; lack of use of any medication known to affect bone metabolism or to harm balance, postural stability, and functional autonomy; and lack of diagnosed or self-reported neurologic disorders, disorders of the vestibular system, and cardiovascular, pulmonary, metabolic, renal, hepatic, or orthopedic medical conditions that contraindicate participation in exercise and testing. On the initial screening visit, all participants received a complete explanation of the purpose, risks, and procedures of the investigation and, after signing a written consent, were interviewed for their past medical history and current medications.

Twelve subjects were excluded due to medical reasons or were unwilling to participate. Sixty subjects were randomly assigned to the exercise-training group ($n = 30$, ET) or control group ($n = 30$, CON), using computer-generated random numbers. The technical assistant who provided the randomization was not involved in the screening, testing, or training procedures. Participants were instructed to continue their daily routines and not to change their PA levels during the course of the experiment. The baseline characteristics of the participants are given in Table 1.

The study was carried out in full compliance with the Helsinki Declaration, and all methods and procedures were approved by the institutional review board.

Measurements

Participants were tested on two occasions: The first assessment was conducted prior to the beginning of training (last week of September 2008) and the second evaluation took place after 8 months of training (first week of June 2009).

Bone and Body Composition

BMD was measured by dual-energy X-ray absorptiometry (DXA) (QDR 4500A; Hologic, Bedford, MA) at the lumbar spine (L1–L4) and the proximal femur on the nondominant side, using standard protocols. To minimize interobserver variation, the same investigator carried out all analyses. Bone phantoms were scanned daily, and

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Table 1 Baseline characteristics of the sample

Variable	Exercise group ($n = 30$)	Control group ($n = 30$)	P^a
Age (years)	70.1 ± 5.4	68.2 ± 5.7	0.095
Age at menarche (years)	13.3 ± 1.7	12.7 ± 1.2	0.223
Age at menopause (years)	47.8 ± 5.1	48.2 ± 2.9	0.628
Married (%)	60.0	56.7	0.793
Education (years)	7.9 ± 3.5	7.3 ± 3.7	0.374
BMI (kg/m^2)	28.4 ± 3.7	28.2 ± 3.7	0.836
Number of routine medications	2.6 ± 1.2	2.5 ± 1.6	0.982
History of (%)			
Hypertension	43.3	40.0	0.793
Diabetes mellitus	3.3	10.0	0.612
Arthritis	26.7	20.0	0.542
Cigarette smoking	10.0	6.7	1.000
Taking lipid-lowering agents (%)	30.0	26.7	0.774
Lactase persistence (%)	87.5	46.7	0.004
Energy intake (kcal/day)	1,428.4 ± 349.6	1,543.3 ± 314.0	0.197
Protein intake (g/day)	60.8 ± 12.3	67.6 ± 14.0	0.052
Calcium intake (mg/day)	654.9 ± 200.8	625.7 ± 265.6	0.644
Phosphorus intake (mg/day)	1,019.6 ± 291.2	998.2 ± 278.0	0.778
Vitamin D intake (μg/day)	1.6 ± 1.2	1.9 ± 2.0	0.987
Coffee intake (mL/day)	43.6 ± 37.8	50.2 ± 58.0	0.786
Time spent in MVPA (minday)	83.8 ± 35.2	79.6 ± 37.8	0.666
Daily count (min^{-1})	376.3 ± 116.9	359.9 ± 148.0	0.648
Daily step count	8,455.3 ± 2,354.1	7,768.3 ± 3,211.3	0.121
Lumbar spine, T score	–1.6 ± 1.1	–1.6 ± 0.8	0.992
Femoral neck, T score	–1.4 ± 0.9	–1.5 ± 0.6	0.485
Total hip, T score	–0.9 ± 1.0	–1.0 ± 0.6	0.709

[a] Student's *t*-test for continuous variables; chi-squared or Fisher's exact test (two-tailed) for categorical variables

coefficients of variation (CV) were verified before and during the experimental period, to ensure assessment reliability.

Total-body scans were taken using the same DXA device. All scans were performed by the same technician using standard procedures as described in the Hologic user's manual. Scans were analyzed for total percent body fat mass.

To test the precision of our DXA scanner, repeated total-body scans were performed on 15 healthy older adults. Each individual underwent three consecutive total-body scans without repositioning. The CV (standard deviation/mean) for repeated measurements was estimated at 0.8% (total body) and those for the femur and spine were 0.9% (neck), 1.1% (total femur), and 0.8% (lumbar spine). CV values for percent body fat, fat mass, and lean body mass were 3.6%, 2.8%, and 1.1%, respectively.

Height and body mass were recorded using a portable stadiometer and balance weighing scales, respectively. Body mass index (BMI) was calculated using the standard formula: mass (kg)/height^2 (m).

Functional Outcomes

Functional performance testing included a 6-min walk test (6MWT) [17], a 30-s chair-stand test (30sCST) [17], and isometric handgrip strength. The 6MWT was performed over a 45-m course within an enclosed level corridor. Participants were asked to walk as fast as possible for 6 min, with verbal encouragement given throughout the test. The score was the total distance walked in 6 min, measured in meters.

Lower-extremity muscle strength was measured using the 30sCST. Participants were asked to sit in a 43-cm-high chair with arms crossed at the wrists and held against the chest. Participants completed as many "stand ups" as possible within 30 s. The score was the total number of stands executed correctly within 30 s.

Grip strength was measured in kilograms in the dominant hand using a digital hand dynamometer (TKK 5401; Grip-D, Tokyo, Japan) with the subjects in a standing position, with the arms at their side and not touching the body throughout the test. Subjects were instructed to grip

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maximally but to squeeze only once for each measurement, and three maximal attempts were recorded, with a 30-s rest in between. If the difference between any two measures was more than 3 kg, then the test was repeated once more after a 2-min rest period. The highest value was used for further analyses.

Muscular Strength

The dynamic concentric muscle strength of the lower extremities (flexion and extension muscle groups) was measured on an isokinetic dynamometer (Biodex System 4 Pro; Biodex, Shirley, NY). Strength measurements were carried out in accordance with the manufacturer's instructions for knee extension/flexion at two angular velocities, 60°/s (1.05 rad/s) and 180°/s (3.14 rad/s). Each participant, after familiarization with the machine, performed five maximal efforts at 180°/s and three at 60°/s with 2 min of rest between tests. The dynamometer angle reading was calibrated to the anatomic joint angle measured by a goniometer, and gravity corrections to torque were based on leg weight at 0° and calculated later by the software of the equipment. Prior to testing, subjects performed a 5-min warm-up on a bicycle ergometer (Bike-Max; Tectrix, Irvine, CA) at 45–60 watts. During the test, participants were encouraged verbally to exert maximal muscular force. The highest value peak torque adjusted to body weight was used for the statistical analyses.

Balance and Mobility Performance Measures

Each subject performed two balance tests: 8-foot Up and Go Test (8ft UG) [17] and one-leg stance (OLS) [18]. Before starting the tests, participants remained seated and resting for 5 min. In the 8ft UG test, the score corresponds to the shortest time to rise from a seated position, walk 2.44 m (8 feet), turn, and return to the seated position, measured to the nearest one-tenth of a second. The OLS test involved standing upright as still as possible in a unipedal stance unassisted (on the nondominant leg) on a 40–60 cm force platform (Force Plate AM 4060-15; Bertec, Columbus, OH) with eyes open, head erect, and arms relaxed by the side of the trunk.

The OLS was timed in seconds from the time one foot was lifted from the floor to when it touched the ground or the standing leg. A longer time indicated better balance ability, and the maximum time was set at 45 s. Two attempts were allowed, with 1 min of rest between; and the best performance was used for force platform–based analysis.

The signals from the force platform were sampled at 500 Hz. We used a personal computer to collect the data with the customized AcqKnowledge-based software (AcqKnowledge 3.9.1; Biopac, Goleta, CA). Data analysis was performed using MATLAB software (MATLAB 7.0; MathWorks, Natick, MA). Data from horizontal forces (Fy and Fx) and center of pressure (COP) time series were low pass–filtered with a zero-lag, fourth-order Butterworth filter with a cut-off frequency of 10 Hz.

The outcome variables were anterior–posterior (AP) and medial–lateral (ML) mean velocity (cm/s) of the COP, and the elliptical area (EA) was calculated using the equation $\sqrt{2}\sigma y \times \sqrt{2}\sigma x$. Mean velocity was determined by dividing the total distance along the signal trajectory by the total recording time.

Lifestyle Behaviors and Clinical Status

A baseline self-administered questionnaire to assess the impact of present and past lifestyle behaviors was completed by interview, to avoid misinterpretations and/or skipped questions. Information regarding education, marital status, falls and fractures history, medical history, actual diseases, medication use, current and past physical activity, menarcheal age, menopause status, current and previous use of hormone-replacement therapy, past dietary habits including calcium intake, and current and past smoking habits was ascertained.

Daily PA

The Actigraph GT1 M accelerometer (Manufacturing Technology, Fort Walton Beach, FL) was used as an objective measure of daily PA, using a 15-s measurement interval (epoch). All participants agreed to wear an accelerometer for 7 consecutive days and were instructed to wear the device over their right hip using an adjustable nylon belt. Exceptions included time spent sleeping and showering. Participants were asked to maintain usual activities. In order for the data to be included in the analyses, participants were required to wear the accelerometer for at least 4 of the 7 days. Two files were corrupt, and four files had only 2 valid days. Those four participants were contacted and agreed to wear the accelerometer again for 7 days (1 week later than the rest of the group). In total, pre- and posttraining data from all participants were included in the analysis (81 files with 7 valid days, 12 files with 6 valid days, and five files with 5 valid days).

The cut point was set at counts per minute $\geq$1,041 (moderate to vigorous PA [MVPA]), which corresponded to a mean VO_2 of 13 mL/kg min, based on the counts associated with a reference activity, which was walking at 3.2 km/hour [19]. Average daily number of MVPA, in at least 10-min bouts, was chosen as the main objective PA outcome as guidelines recommend that older adults should accumulate at least 30 min/day (in bouts of at least 10 min

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each) of moderate-intensity activities or at least 20 min/day of continuous activity of vigorous-intensity activities [20]. Average step-count and daily activity counts per minute were also analyzed.

Nutritional Assessment

Nutritional status was assessed by a 4-day diet records over 3 weekdays and 1 weekend day. To ensure standardization of the dietary records, a dietician instructed the subjects individually on how to fill out the diet records and assess food servings and sizes. Diet records were analyzed using the Food Processor Plus® (ESHA Research, Salem, OR), which uses the table of food components from the U.S. Department of Agriculture. Some traditional Portuguese dishes were added according to the table of Portuguese food composition. The ET and CON groups were compared for intake of total calories, protein, calcium, phosphorus, vitamin D, and caffeine.

Lactose Persistence Status

Lactose persistence mutations C/T -13910 were genotyped by direct sequencing. A 359-bp fragment containing all mentioned mutations and located in intron 13 of the *MCM6* gene was amplified using primers 5′-GCAGGGCTCAA AGAACAATC-3′ (forward) and 5′-TGTTGCATGTTT TTAATCTTTGG-3′ (reverse). PCRs contained 0.5 μM of each primer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 750 mM Tris-HCI (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 1.5 mM $MgCl_2$ and 1 U *Taq* polymerase. The PCR profile consisted of 94°C for 5 min, 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, followed by 20-min extension at 72°C.

Sequencing reactions were carried using the ABI Big Dye v3.1 Ready Reaction Kit and the protocol specified by the manufacturer (Applied Biosystems, Foster City, CA). Products were run on an ABI PRISM 3130x1 sequencer and analyzed in the ABI PRISM 3130x1 Genetic Analyzer software (Applied Biosystems). The resulting chromatograms were inspected for the presence or absence of the lactase mutations using MEGA4.0 software (www.megasoftware.net) [21]. DNA was obtained from buccal swabs using standard extraction methods.

Exercise Intervention

The ET group completed a 32-week progressive multicomponent training consisting of two sessions per week. Each session lasted about 60 min, and all sessions were accompanied by appropriate music considered relevant to the required activity and participants' age. The exercise training was designed to load bones with intermittent and multidirectional compressive forces, introducing atypical and novel stress on the bone, and to improve neuromuscular function. Each training session included six components: a 10-min light stretching and warm-up exercise; 15 min of weight-bearing activities, consisting of moderate- to high-impact activities such as marching in place, stepping exercise at a speed of 120–125 beats per minute using a 15-cm-high bench, and heel-drops performed on a hard surface. A heel-drop consists of raising the body weight onto the toes and then letting it drop to the floor, keeping the knees locked and hips extended. The estimating loads based on calculations of the GRF for the heel-droop exercise are illustrated in Fig. 1; muscular endurance exercises performed concentrically and eccentrically for about 10 min, involving squats while wearing weight vests, hip flexors, extensors, and abductors; knee flexors and extensors and upper body exercises performed using elastic bands and dumbbells; 10 min of balance training with static and dynamic exercises (e.g., walking in a straight line, walking heel to toe, using additional resources such as ropes, sticks, balls, and balloons), 10 min of agility training aimed at challenge hand–eye coordination, foot–eye coordination, dynamic balance, standing and leaning balance, and psychomotor performance (reaction time) including ball games, relay races, dance movements, and obstacle courses; and 5 min of stretching. For weight-bearing and strength exercises, the repetitions were increased from eight to 15 and the number of sets increased to three. Each session was led by two physical education instructors specialized in PA for older adults and supervised by the researchers.

Peak GRF was measured using a force platform (Bertec Force Plate AM 4060-15) in a randomly selected

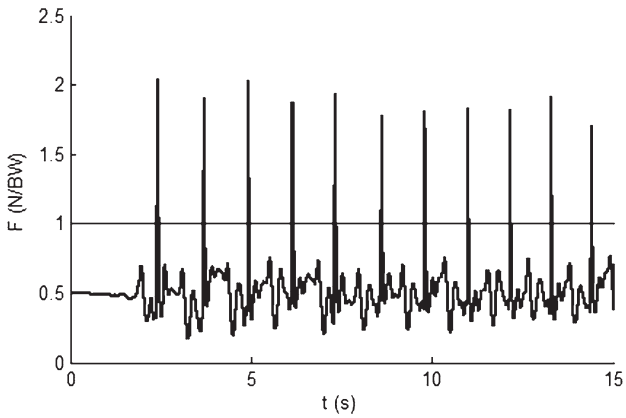

Fig. 1 Ground reaction forces (GRFs) recorded during heel-drops performed with shoes by a 72-kg woman. *N/BW* Newtons/body weight

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subsample of volunteers ($n = 10$). The force platform was used to record GRF with a 500 Hz sampling rate. Values for the peak force on the vertical axis were then obtained from the recordings at take-off and landing. The average GRF was 1.7 times body weight (BW) for marching, 1.8 times BW for step basic movement from a 15-cm bench, and 2.7 times BW for heel-drop.

Exercise compliance was defined as the number of exercise sessions reported divided by the number of maximum exercise sessions possible.

Statistical Analysis

All statistical analyses were performed using PASW Statistics (version 18; SPSS, Inc., Chicago, IL) for Windows with a significance level of 0.05. Data were checked for distribution, and the means ± SD were calculated. Primary outcomes were changes from baseline in response to the 8-month intervention in balance, muscle strength, and BMD. Secondary outcomes included 8-month changes from baseline in dietary intake, daily PA, 6MWT, handgrip, body composition (BMI, waist circumference, fat, fat-free mass, and lean mass), and the presence or absence of the lactase mutations. The results were first analyzed on an intention-to-treat (ITT) basis, and missing data due to lack of follow-up (the method assumed data were missing at random) were replaced using the process of multiple imputation. This method has been adapted to the analysis of longitudinal data [22]. The secondary analysis was a per-protocol analysis using data from all individuals who completed the trial (efficacy subset analysis). Between-group comparisons of continuous variables were performed using *t*-tests, except for a few cases where required conditions were not satisfied, and the Wilcoxon test was used as a nonparametric alternative. Chi-squared tests were used for between-group comparisons of categorical variables or for differences in proportions at baseline, except when cell sample sizes in the contingency table were small, in which case Fisher's exact test was used.

Pearson correlations were used to determine the relationship of potential confounding variables (e.g., lactase persistence, PA change, fat mass change) with primary outcomes. The delta percentage was calculated with the standard formula: % change = [(posttest score − pretest score)/pretest score] × 100. Two-way ANOVA (2 times [initial and final] × 2 groups [exercise and control]) with repeated measures was used to determine differences between and within the ET and CON groups for dependent variables. Main effects were considered when interactions were not significant. When ANOVA revealed significant interaction (time × group), Bonferroni post hoc tests were performed to determine differences between initial and final values in each group.

A power analysis based on a formulation of 80% power, an effect size of 0.5 for overall muscle strength, balance and BMD from previous studies, and a significance level of 0.05 for a one-tailed test deemed that a sample of 54 subjects (27 per group) was sufficient to address the research questions.

Results

Recruitment

Of the 72 participants interested in participating, 12 were excluded because of loss of interest or inability to commit (9/12, 75%). Thus, from the 60 subjects aged 69.9 ± 5.8 (range 63–83) who underwent the initial assessment and randomization, 49 completed the study. Two participants discontinued the intervention because of surgery and two due to medical issues unrelated to intervention. Three participants left the study due to unwillingness to participate, and four indicated personal reasons. No differences ($P = 0.095$) in dropout rates were observed between groups. Figure 2 shows the number of participants at each stage of the study.

Subject Characteristics

Demographics and descriptive parameters of the subjects (30 ET, 30 CON) are listed in Table 1. On average, participants were overweight, most of them suffered hypertension, and a small portion had a history of cigarette smoking. On average, participants obtained 82 min of MVPA per day and had a similar intake of nutrients in both groups. There were no significant group differences in any baseline characteristic with the exception of lactase persistence (with *TT/TC* genotypes), where the prevalence was higher for ET than CON. The prevalence of the *TT* and *TC* genotypes was 87.5% and 46.7%, respectively.

Compliance with Intervention and Adverse Events

One-hundred percent compliance to the exercise sessions was set at 64 training sessions. Median compliance to the exercise sessions was 68.3% (34.4–85.9%) including the three dropouts and 72.4% (51.6–85.9%) excluding dropouts. There were no exercise- or assessment-related (pre- and posttraining) adverse events.

In comparison to individuals who completed the trial, those who failed to provide follow-up data had no significant differences in any baseline measurements, including age, body weight, daily MVPA levels, strength, balance, or BMD.

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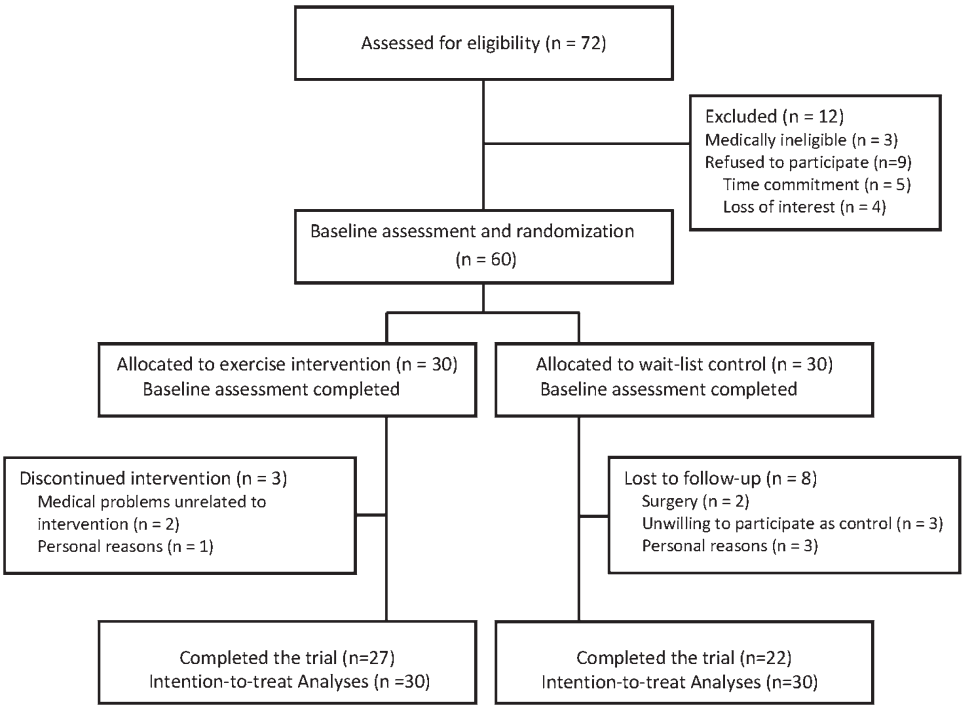

Fig. 2 Flow of participants through the study

Dietary Intake

Total energy intakes were similar among the groups at baseline and after 8 months. Energy intake was 1,489 ± 333 kcal/day at baseline and 1,528 ± 244 kcal/day at 8 months ($P > 0.05$ for both group changes). No group differences were apparent in baseline values (Table 1) or change in dietary protein, phosphorus, caffeine, calcium, and vitamin D intake in response to the interventions (data not shown). No significant difference in mean daily total calcium intake derived from milk and dairy products was evident among lactase persistents and restrictors within each group.

Daily PA

No significant changes in MVPA level were observed at 8 months. There was no significant main effect ($P = 0.256$ and $P = 0.060$ for group and time, respectively) or interaction ($P = 0.186$) between time and group treatments on PA changes. Change in MVPA was not related to changes in the primary outcomes.

Changes in Body Composition and Functional Fitness Variables

At baseline, there were no significant group differences with respect to physical characteristics. Results from the ITT analysis ($n = 60$) determined that there were significant group × time interactions on fat mass and handgrip (Table 2). Accordingly, for those variables a different response in each group was evident over time. Indeed, only the ET group significantly decreased fat mass and increased handgrip strength. There was a significant main effect of time on waist circumference and 6MWT ($P < 0.001$ and $P = 0.039$, respectively). There were no significant interactive or main effects of time and group on changes in BMI, lean mass, and fat-free mass.

Results from the per-protocol analysis ($n = 49$) on exercise effects on changes in body composition were similar in direction, but the statistical significance changed. Similar to the ITT analysis, there was a significant interaction of handgrip and fat mass ($P = 0.030$ and $P < 0.001$, respectively) and there were no significant interactions or main effects on changes in BMI, lean mass, and fat-free mass. However, there was a significant interaction on waist circumference ($P = 0.004$), and the main effect of time ($P = 0.492$) on 6MWT was no longer present. Moreover, although not significant, the changes in BMI were divergent in direction compared to the ITT analysis. BMI slightly increased in the CON group at 8 months. Changes in lean, fat-free, and fat mass were not related to changes in balance strength and BMD.

Changes in Balance

The results for balance tests and variables of postural sway are presented in Table 3. Using the ITT analysis ($n = 60$), significant group × time interactions were found on stance test time and velocity values for both the AP and ML

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Table 2 Pre- and posttraining values for body composition and functional fitness variables

Variable	Exercise group ($n = 30$)		Control group ($n = 30$)		P (group)	P (time)	P (interaction)
	Pretraining	Posttraining	Pretraining	Posttraining			
BMI (kg/m^2)	28.4 ± 3.7	28.4 ± 3.3	28.2 ± 3.7	27.7 ± 2.5	0.571	0.490	0.337
WC (cm)	91.3 ± 6.5	86.6 ± 5.3	90.7 ± 7.7	87.0 ± 7.1	0.942	<0.001	0.516
Fat mass (%)	38.0 ± 2.9	35.9 ± 3.1*,**	37.7 ± 4.4	37.9 ± 3.1	0.366	0.006	<0.001
Lean mass (kg)	39.3 ± 5.5	40.6 ± 5.8	40.6 ± 5.3	40.6 ± 4.0	0.596	0.182	0.172
Fat-free mass (kg)	41.1 ± 6.0	42.4 ± 6.0	42.5 ± 5.6	42.6 ± 4.3	0.565	0.165	0.184
6MWT (m)	544.8 ± 67.4	555.0 ± 62.0	515.0 ± 68.4	535.5 ± 67.3	0.130	0.039	0.483
Handgrip (kg)	24.7 ± 4.2	27.0 ± 3.2*,**	24.8 ± 3.9	25.4 ± 1.3	0.312	0.005	0.049

BMI body mass index, *WC* waist circumference, *6MWT* 6-min walking test

* Significant difference from CON $P < 0.05$; ** Significant intragroup difference $P < 0.05$

Table 3 Pre- and posttraining values for dynamic and static balance tests

Variable	Exercise group ($n = 30$)		Control group ($n = 30$)		P (group)	P (time)	P (interaction)
	Pretraining	Posttraining	Pretraining	Posttraining			
8ft UG (s)	5.8 ± 1.2	5.0 ± 0.8	6.3 ± 1.2	5.9 ± 0.9	0.004	<0.001	0.228
OLS (s)	28.3 ± 14.7	34.2 ± 12.5*	33.5 ± 14.6	31.2 ± 12.3	0.741	0.287	0.020
EA (cm^2)	9.8 ± 9.2	10.4 ± 10.4	4.9 ± 3.0	3.8 ± 1.5	<0.001	0.852	0.494
AP velocity (cm/s)	3.9 ± 0.9	3.2 ± 0.8*	3.0 ± 0.7	2.9 ± 0.4	0.001	<0.001	0.002
ML velocity (cm/s)	4.5 ± 1.7	3.8 ± 1.8*	2.8 ± 1.0	3.3 ± 0.8	0.001	0.486	0.004

8ft UG 8-foot Up and Go Test, *OLS* one leg stance, *EA* elliptical area, *AP* anterior–posterior, *ML* medial–lateral

* Significant intragroup difference $P < 0.05$

directions. Thus, only the ET group significantly increased the time of performance and decreased the mean velocity of the COP displacement, and no significant changes were observed for the CON group. There were significant main effects of group ($P = 0.038$) and time ($P = 0.003$) on dynamic balance test and a significant main effect of group on EA of the COP.

Using the per-protocol analysis ($n = 49$), the results for balance tests and variables of postural sway were similar in direction, except EA where both groups inverted the direction of change, although no significant interactive ($P = 0.437$) or main effects occurred. Similar to the ITT analysis, there were significant interactions between group and time on stance test time and velocity values for both the AP and ML directions and significant main effects of group ($P = 0.038$) and time ($P = 0.003$) in the dynamic balance test. In addition, significant differences in percent changes in dynamic balance (–11.9% vs. –2.7% for ET and CON, respectively) and EA of the COP (–15.3% vs. 17.2% for ET and CON, respectively) were observed.

Changes in Strength

At baseline, there were no significant differences among groups in muscle strength at any site measured or test. Table 4 shows the results for muscle strength on maximal knee extension and knee flexion torques adjusted for body weight and for 30sCST analyzed on an ITT basis. As seen, the ET group showed a clear trend to improve (mean change 2.24–9.09 Nm) in all maximal strength tests for both legs, whereas the CON group demonstrated an irregular directional change. Results from the ITT analysis ($n = 60$) determined that there were significant group × time interactions on knee flexion torque for the left leg at 180°/s. Thus, different responses in the ET and CON groups were evident over time, supported by the significant increase for the ET group. An overall time effect was found on knee extension torque for the right leg at 180°/s ($P = 0.001$) and on knee flexion torque for the right ($P = 0.002$) and left ($P = 0.009$) legs at 60°/s. Also, a significant main effect of time in the 30sCST was observed where both groups had increased 30sCST

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Table 4 Muscle strength at baseline and after the 8-month intervention period

Variable	Exercise group ($n = 30$)		Control group ($n = 30$)		P (group)	P (time)	P (interaction)
	Pretraining	Posttraining	Pretraining	Posttraining			
30sCST (rep)	17.3 ± 4.4	19.8 ± 3.8	15.5 ± 3.6	17.7 ± 5.0	0.055	<0.001	0.801
KE (PT/BW L leg 180°/s)	82.9 ± 20.6	90.9 ± 20.0	81.1 ± 26.0	92.0 ± 19.4	0.949	0.001	0.595
KE (PT/BW L leg 180°/s)	77.6 ± 18.6	79.8 ± 24.5	80.3 ± 19.5	84.6 ± 19.7	0.396	0.325	0.757
KF (PT/BW L leg 180°/s)	51.1 ± 15.4	55.1 ± 16.1	50.2 ± 16.1	53.0 ± 9.5	0.662	0.083	0.760
KF (PT/BW L leg 180°/s)	49.9 ± 14.0	56.8 ± 14.4*	51.5 ± 14.9	51.1 ± 10.7	0.536	0.047	0.027
KE (PT/BW R leg 60°/s)	137.6 ± 27.5	141.9 ± 34.8	142.7 ± 42.7	140.7 ± 33.8	0.822	0.767	0.407
KE (PT/BW L leg 60°/s)	126.5 ± 36.5	135.5 ± 30.9	134.1 ± 31.9	132.9 ± 31.9	0.738	0.365	0.239
KF (PT/BW R leg 60°/s)	68.9 ± 19.1	75.1 ± 20.2	69.9 ± 21.9	77.8 ± 25.8	0.739	0.002	0.707
KF (PT/BW L leg 60°/s)	72.5 ± 18.0	79.5 ± 20.5	71.7 ± 20.6	73.9 ± 19.3	0.515	0.009	0.163

Rep number of repetitions, *30sCST* 30-s chair stand test, *KE* knee extension, *PT* peak torque, *BW* body weight, *R* right, *L* left

* Significant intragroup difference $P < 0.05$

performance. In addition, no significant changes ($P > 0.050$) were observed following 8 months for all strength tests in the CON group.

Results from the per-protocol analysis ($n = 49$) on changes in muscle strength were similar in direction for the ET group, but the statistical significance changed. Similar to the ITT analysis, there were significant main effects of time on knee extension torque for the right leg at 180°/s ($P = 0.002$), on knee flexion torque for the right ($P = 0.005$) and left ($P = 0.034$) legs at 60°/s, and on 30sCST ($P < 0.001$). However, the significant interaction between time and group on knee flexion torque for the left leg at 180°/s was no longer apparent. On the other hand, significant group × time interactions were found on knee extension ($P = 0.005$) and flexion ($P = 0.022$) torques for the left leg at 60°/s, supporting the significant increase for the ET group on both isokinetic tests.

Changes in BMD

At baseline there were no significant differences among groups in BMD at any site measured. Using the ITT analysis ($n = 60$), there was a significant interaction of group × time in BMD at the femoral neck ($P = 0.008$) (Table 5). Thus, femoral neck BMD significantly increased by 0.018 ± 0.028 g/cm^2 (2.8% ± 4.6%) in the ET group, with a nonsignificant trend to increase BMD at all other sites. No significant changes were observed for all measured sites in the CON group ($P > 0.05$). Moreover, because no associations were found between lactase nonpersistence and BMD changes, such a confounding variable was not entered as a covariate into the analysis of variance model. Using the per-protocol analysis ($n = 49$) did not alter the BMD results, with a significant interaction of group × time in BMD at the femoral neck ($P = 0.010$), and the other effects for the additional bone sites remained nonsignificant.

Discussion

The present study tested the hypothesis that 8 months of multicomponent training with weight-bearing exercises is effective at increasing BMD, muscle strength, and distinct functional fitness skills, which are associated with aging and increased risk of falling and fracture. These improvements were found to be independent of known influence factors such as PA levels, dietary intakes, and lactase nonpersistence as no significant changes and associations were found with the main outcomes. Our hypothesis was confirmed as femoral neck BMD, muscle strength, and balance improved after the training period. MVPA levels in the ET group were not significantly different from those of the CON group after 32 weeks of training, suggesting that this training protocol had per se an important modulator role on bone and neuromuscular adaptations.

Falls prevention associated with positive adaptations of muscle strength, flexibility, and balance has previously been studied [23]. The present work confirmed that exercise results in increased muscle strength and balance, reinforcing the exercise potential to reduce fall risk and promote functional independence [24].

We observed a significant main effect of time in 30sCST performance and a significant increase in knee flexion torque for the left leg at 180°/s in the ET group. These data are in accordance with other reports using multicomponent protocols [25, 26]. Improvements in handgrip strength are especially important in the elderly, in whom weak grip strength has been shown to be a significant predictor of recurrent falls [27] and increased

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Table 5 Eight-month changes for BMD

Variable	Exercise group ($n = 30$)		Control group ($n = 30$)		P (group)	P (time)	P (interaction)
	Pretraining	Posttraining	Pretraining	Posttraining			
Femoral neck (g/cm^2)	0.699 ± 0.091	0.717 ± 0.085*,**	0.678 ± 0.061	0.671 ± 0.051	0.084	0.223	0.008
Troch (g/cm^2)	0.621 ± 0.078	0.628 ± 0.081	0.625 ± 0.047	0.628 ± 0.034	0.894	0.233	0.557
Inter (g/cm^2)	0.986 ± 0.143	0.989 ± 0.148	0.981 ± 0.107	0.977 ± 0.075	0.785	0.925	0.686
Total femur (g/cm^2)	0.828 ± 0.103	0.832 ± 0.104	0.822 ± 0.071	0.823 ± 0.058	0.734	0.638	0.672
Lumbar spine (g/cm^2)	0.857 ± 0.097	0.868 ± 0.094	0.868 ± 0.080	0.863 ± 0.065	0.911	0.696	0.233

BMD bone mineral density, *Troch* trochanter, *Inter* intertrochanteric region

* Significant difference from CON $P < 0.05$; ** Significant intragroup difference $P < 0.05$

risk of fracture [28]. The data confirm that multicomponent exercise improves handgrip strength, as previously observed [12].

There is general agreement that exercise can reduce fall rates in older people [24] and multicomponent interventions involving balance and coordination training appear to have the greatest impact on balance ability [4, 29], which corroborates the present data. To better understand integrated functioning of the balance control systems, we used force platform–based measures to acquire detailed information regarding control processes and biomechanical changes relevant to balance. The present study showed that participants assigned to the ET group had significantly better sway measures, namely, average velocity of the COP, relative to the CON group. Accordingly, our data are in accordance with other intervention studies involving balance, coordination, and/or functional exercises [23, 30]. Moreover, in line with other reports [23, 25], dynamic balance also improved after training. In this regard and considering the contribution of lower limbs to the testing, we could hypothesize that the observed balance improvements after the exercise program would reflect and/or be influenced, at least partially, by the increase in muscle strength. Thus, the interplay between exercise and falls prevention can be exemplified by the evident improvement of physical fitness associated with better muscle strength and balance.

Although improvements in 6MWT performance and body composition have not been consistently related with the positive effect of exercise on falls and bone fracture prevention, the enhancement of those function-related parameters seems beneficial from the standpoint of contributing to successful aging. Exercise training resulted in a significant decrease in body fat mass, although BMI remained constant. On the other hand, no significant increase in 6MWT performance was found. Because the present experimental protocol was designed to improve skeletal integrity and neuromuscular function, the lack of a marked endurance component seemed to compromise walking performance improvement in older women. In fact, multicomponent exercise including endurance training has proven to positively modulate aerobic endurance capacity [31].

Bone is inherently mechanosensitive, which implies that the external forces imposed upon the bone via exercise need to be of a sufficient magnitude to create a fluid flow within the lacunar–canalicular network to stimulate bone formation [32]. Our data regarding the estimates of the applied loading to the skeleton with the specific weight-bearing exercises suggest that moderate-impact forces (average GRF range 1.7–2.7 times BW) results in significant changes in BMD at the femoral neck. Because bone tissue adapts to joint and GRF in a dose-dependent manner and mechanical loading creates strains which are linearly proportional to the applied loads in the bones [33], higher increments in BMD would be expected for higher loading impact forces. Of note, exercises that introduce high strain levels to the skeleton are difficult to perform with increasing age due to the high risk of traumatic fractures, stress injuries, and arthritic complications. Moreover, several potential sources of skeletal structural failure are coupled with the aging process, such as progressive sarcopenia, altered balance of formative and resorptive activity, genetic predisposition to reduced BMD, microdamage, and corrosion of the organic matrix [34, 35]. In fact, a much larger body of data from clinical studies of BMD provides information about how pre- and postmenopausal women, mostly aged between 40 and 60 years, respond to training. Several studies frequently included high-impact exercises such as vertical jumping, producing GRFs equivalent to two to six times the body weight per jump [36, 37]. Results have been equivocal, demonstrating that mechanical stress can enhance bone mass in premenopausal women [38] or have no effect in postmenopausal women [39]. Conversely, no studies on exercise interventions focused on older adults have estimated loadings based on calculations of GRF. Although in the present study the magnitude of the applied loads was

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smaller than in the above-mentioned studies on pre- and postmenopausal women, our mechanical loading seemed to be sufficient to improve femoral neck BMD in older women. Our results on the multicomponent training-induced elevation in BMD are consistent with prior studies that have similarly reported on the effectiveness of exercise in promoting an osteogenic response in the elderly [12, 30, 40]. However, there remains some controversy regarding its osteogenic potential in older adults. Hourigan et al. [23] reported no significant alterations in BMD at the proximal femur or lumbar spine after 20 weeks of balance training and weight-bearing exercise. Similarly, Stewart et al. [41] showed that a combined program including resistance and aerobic exercises performed three times per week for 24 weeks had no effect on BMD among men and reduced BMD among women. Considering that the time taken for completion of the bone remodeling cycle (bone resorption, formation, and mineralization) is around 3–4 months [42], training should be held at least for 6–8 months to ensure that the potential changes in structural properties are attained. In the present study we detected a significant mean 0.018 g/cm^2 (2.8%) increase in femoral neck in the ET group, while in the CON group a modest decrease of 0.007 g/cm^2 (–0.7%) was observed. From a clinical point of view, a decrease of 0.110 g/cm^2 in femoral neck BMD in older women was associated with a 2.6-fold increase in relative risk for hip fracture [43]. Therefore, and considering that low femoral neck bone density is a stronger predictor of hip fracture than bone density at other sites [43], it seems probable that the increase in BMD reported here following multicomponent training might represent a relevant decrease in relative risk for hip fracture.

Nutritional variables (such as protein, phosphorus, caffeine, calcium, and vitamin D intake) can independently affect bone material properties [44]. Our dietary assessment revealed a nonsignificant change in diet patterns, and no significant association with changes on BMD were found. This lack of change and association emphasize the leading exercise osteogenic potential. Although lactase deficiency has been associated with BMD [14] and reduced intake of calcium [45] could also represent a genetic risk factor for bone fractures in older adults [16], no associations were found between lactase deficiency status and reduced BMD and calcium intake or BMD changes after training.

A major limitation of this study is the lack of concurrent measures of biochemical markers of bone turnover. During the remodeling process, osteoblasts synthesize a number of cytokines, peptides, and growth factors that are released into the circulation; and their concentration thus reflects the rate of bone formation. On the other hand, the mechanical competence of bone is a function not only of its intrinsic material properties (mass, density, and stiffness) but also of its structural properties (size, shape, and geometry). DXA is the method most commonly used to measure areal BMD because of its speed, precision, low radiation exposure, and availability of reference data [46]; but this two-dimensional skeletal outcome represents only one part of overall bone strength.

A key strength of our study is that, to our knowledge, it may be the first study that has considered the possible influence of critical confounding variables, such as daily PA levels, nutrition, and lactose persistence (genetically defined by the C/T -13910 polymorphism). Moreover, the variation of loads in the weight-bearing exercises was estimated based on calculation of the GRF. In addition, we used force platforms to document the biomechanical changes of balance, which has been scarcely used to assess postural balance in older people.

In conclusion, the data presented here lend support to our preliminary hypothesis that multicomponent training with moderate-intensity weight-bearing exercise can create significant bone adaptation over an 8-month time course in postmenopausal older women. We have also demonstrated that the exercise training regimen used in this study elicited gains in muscle strength and balance. Although these findings provide some clue to the exercise potential to reduce fracture risk in community-dwelling older women, additional evidence is needed to validate and build upon our findings using additional outcome measures.

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Paper III

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Effects of resistance and aerobic exercise on physical function, bone mineral density, OPG and RANKL in older women

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ABSTRACT

This study compared the effects of a resistance training protocol and a moderate-impact aerobic training protocol on bone mineral density (BMD), physical ability, serum osteoprotegerin (OPG), and receptor activator of nuclear factor kappa B ligand (RANKL) levels. Seventy-one older women were randomly assigned to resistance exercise (RE), aerobic exercise (AE) or a control group (CON). Both interventions were conducted 3 times per week for 8 months. Outcome measures included proximal femur BMD, muscle strength, balance, body composition, serum OPG, and RANKL levels. Potential confounding variables included dietary intake, accelerometer-based physical activity (PA), and molecularly defined lactase nonpersistence. After 8 months, only RE group exhibited increases in BMD at the trochanter (2.9%) and total hip (1.5%), and improved body composition. Both RE and AE groups improved balance. No significant changes were observed in OPG and RANKL levels, and OPG/RANKL ratio. Lactase nonpersistence was not associated with BMD changes. No group differences were observed in baseline values or change in dietary intakes and daily PA. Data suggest that 8 months of RE may be more effective than AE for inducing favourable changes in BMD and muscle strength, whilst both interventions demonstrate to protect against the functional balance control that is strongly related to fall risk.

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1. Introduction

Low bone mass and an increased risk of fracture rank high amongst the serious clinical problems faced by older adults. As such, the impact of this age-related condition extends beyond the significance of an increased prevalence, as severe individual and economic consequences of injurious falls can have profound implications for subsequent health, morbidity, functional independence, life quality and increased mortality of older people (Lane, 2006).

Many risk factors for falls have been identified, and increasing evidence has suggested that fall reduction programmes that involve systematic fall risk assessment and targeted interventions, exercise programmes and environmental and hazard-reduction programmes are the optimal approaches (Rubenstein, 2006). Importantly, lower extremity weakness as well as power and balance impairment is frequently reported as a risk factor that has the potential to be influenced with appropriate exercise prescription (Sherrington et al., 2008). As exercise may be an important way to reduce the incidence of this problem, recent systematic reviews have consistently shown that exercise can be used as a stand-alone intervention for fall prevention (Sherrington et al., 2008; Gillespie et al., 2009). Despite the positive effects seen in programmes that include strengthening, balance, and/or endurance training (Sherrington et al., 2008), and the benefit of aerobic exercise training (AE) or resistance exercise training (RE) as single interventions remains controversial, due mostly to the paucity of data. In fact, both types of activity are commonly prescribed and widely accepted, based on the variety of favourable adaptations that AE and RE in isolation can elicit in older adults (Chodzko-Zajko et al., 2009). Although the evidence that supports the notion that older adults can significantly increase their muscle strength and power after RE are overwhelming (Chodzko-Zajko et al., 2009), currently published data have not consistently shown that the use of RE alone improves balance in this population (Orr et al., 2008).

Abbreviations: AE, aerobic exercise; ANOVA, one-way analysis of variance; AP, anterior–posterior; BMD, bone mineral density; CON, control group; COP, centre of pressure; CV, coefficient of variation; EA, elliptical area; ELISA, enzyme-linked immunosorbent assay; KE, knee extension; KF, knee flexion; ML, medial–lateral; MVPA, moderate to vigorous physical activity; OLS, one-leg stance; OPG, osteoprotegerin; PA, physical activity; RANKL, receptor activator of nuclear factor kappa B ligand; RE, resistance exercise; 8-ft UG test, 8-foot Up and Go Test.

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Nevertheless, AE has been highlighted as the exercise regimen of choice for inducing several cardiovascular adaptations (Chodzko-Zajko et al., 2009); however its effectiveness in increasing muscle strength and balance is still under discussion.

In addition to its role in the prevention of falls, exercise is also considered to play a crucial role in bone modelling and remodelling (Borer, 2005). Animal studies have evaluated osteogenic responses to several exercise interventions, including running, swimming, jumping, climbing, and resistance training (Warner et al., 2006; Mori et al., 2003; Notomi et al., 2000). The results suggest that the exercise-induced osteogenic effect is site specific and dependent on the type of exercise and load applied to the bones. Most of the literature, usually based in animal models, published thus far supports the notion that greater strain magnitudes and unusual strain distributions provide the most effective stimuli for bone formation (Bailey and Brooke-Wavell, 2008). In support of this, RE has been recognised to be effective in stimulating an osteogenic response and elevating bone mineral density (BMD) in both young and old adults (Ryan et al., 2004). However, the isolated effects of AE on bone mass in older adults have been poorly investigated. Evidence regarding the effectiveness of this type of exercise in counteracting age-related declines in BMD has been controversial (Brooke-Wavell et al., 2001; Bonaiuti et al., 2002). Notably, the data suggest that the skeletal response to exercise is altered with age (Lanyon and Skerry, 2001). Actually, mechanical loading forces become less effective in eliciting an osteogenic effect with increasing age, suggesting a progressive loss of bone sensitivity to chemical and physical signals (Rubin et al., 1992). In addition, basic and clinical studies have established a consistent relationship between the osteoprotegerin (OPG)/receptor activator of the nuclear factor-kB (RANK)/RANK ligand (RANKL) system and skeletal health due to its critical role in bone remodelling (Boyce and Xing, 2008). OPG has an osteo-protective role in humans, protecting bones from excessive bone resorption via binding to RANKL and preventing it from binding to RANK (Boyce and Xing, 2008). In vitro and in vivo experiments have shown that mechanical stimulation can inhibit osteoclast formation and activity by changing the OPG/RANKL ratio in favour of OPG (Saunders et al., 2006; Rubin et al., 2003). However, the association between serum OPG/RANKL and the incidence of bone fractures and BMD has been inconsistent (Browner et al., 2001; Jorgensen et al., 2004; Stern et al., 2007). Although there is a great deal of basic research currently addressing the RANKL/RANK signalling pathway, less is known regarding how prolonged exercise may influence the release of soluble factors and if this reflects what is happening in bones. The present study aimed to compare the alterations in key factors associated with fracture risk, namely BMD, muscle strength and balance. We also tested whether there are changes in serum levels of OPG, RANKL and their ratios alter after an 8-month exercise training programme. The results obtained would contribute to a better understanding of how different exercise interventions can interact with the physiological systems associated with bone turnover and remodelling.

2. Materials and methods

2.1. Subjects and experimental design

Subjects were recruited through advertisements in Porto area newspapers for participation in this university-based study. A total of 90 Caucasian older women volunteered to participate in the study. The eligible subject pool was restricted to older women with the following characteristics: free of hormone therapy use for at least two years, aged 60–95 years, community-dwelling status, not engaged in regular exercise training in the last year, lack of use of any medication known to affect bone metabolism or to harm balance, postural stability and functional autonomy; and lack of diagnosed or self-reported neurologic disorders, disorders of the vestibular system, and cardiovascular, pulmonary, metabolic, renal, hepatic, or orthopaedic medical conditions that contraindicate participation in exercise. On the initial screening visit, all participants received a complete explanation of the purpose, risks, and procedures of the investigation and, after signing a written consent form, the past medical history and current medications of the subjects were determined. Nineteen subjects were excluded due to medical reasons, inability to be contacted or lack of willingness to participate in the study. Seventy-one subjects were randomised into one of three groups: resistance exercise training (n = 23, RE), aerobic exercise training (n = 24, AE), and a control group (n = 24, CON), using computer-generated random numbers. The technical assistant who provided the randomisation was not involved in the screening, testing, or training procedures. Participants were instructed to continue their daily routines and to refrain from changing their physical activity (PA) levels during the course of the experiment.

The baseline characteristics of the participants are given in Table 1. The study was carried out in full compliance with the Helsinki Declaration, and all methods and procedures were approved by the institutional review board.

2.2. Measurements

Participants were tested on two occasions: the first assessment was conducted prior to the beginning of training and the second evaluation took place after eight months of training.

Table 1
Baseline characteristics of the sample.

Variable	RE group (n = 23)	AE group (n = 24)	CON group (n = 24)	p-value[a]
Age, y	67.3 ± 5.2	70.3 ± 5.5	67.9 ± 5.9	0.17
Age at menarche, y	13.3 ± 1.2	13.7 ± 1.4	12.8 ± 1.3	0.08
Age at menopause, y	47.6 ± 3.5	48.3 ± 5.2	48.7 ± 3.6	0.86
Married, %	63.4	55.0	73.7	0.48
Education, y	9.1 ± 4.9	8.4 ± 3.4	7.4 ± 4.3	0.54
BMI, kg/m^2	28.8 ± 4.6	27.5 ± 3.8	28.1 ± 3.5	0.61
Total body fat, %	38.8 ± 4.4	39.2 ± 4.5	38.4 ± 4.6	0.84
Waist circumference, cm	93.0 ± 10.5	89.1 ± 9.5	91.4 ± 8.7	0.38
Number of routine medications	1.8 ± 1.8	2.7 ± 2.0	2.6 ± 1.6	0.42
History of, %				
Hypertension	45.5	40.0	63.2	0.33
Diabetes mellitus	9.1	10.0	15.8	0.81
Arthritis	9.1	10.0	15.8	0.81
Cigarette smoking	18.2	10.0	10.5	0.77
Taking lipid-lowering agents, %	27.3	5.0	21.1	0.20
Lactase persistence, %	42.9	68.4	45.5	0.18
Energy intake, kcal/day	1485.7 ± 360.3	1368.2 ± 241.4	1561.0 ± 334.1	0.13
Protein intake, g/day	65.6 ± 13.9	64.5 ± 15.4	69.5 ± 15.2	0.52
Calcium intake, mg/day	714.5 ± 358.4	608.5 ± 248.8	636.9 ± 280.9	0.54
Phosphorus intake, mg/day	1013.5 ± 307.8	965.4 ± 237.3	979.2 ± 273.0	0.86
Vitamin D intake, μg/d	1.8 ± 1.7	2.3 ± 1.4	2.0 ± 1.9	0.39
Coffee intake, mL/day	66.5 ± 53.5	48.7 ± 41.5	43.7 ± 50.9	0.37
Time spend in MVPA, min/day	93.2 ± 26.3	86.2 ± 32.1	78.8 ± 40.5	0.43
Daily count min^{-1}	412.6 ± 117.9	355.6 ± 113.0	360.8 ± 161.6	0.39
Daily step count	9000.9 ± 2544.6	8852.7 ± 2306.4	7905.1 ± 3323.1	0.40
Femoral neck, T-score	−1.6 ± 0.7	−1.8 ± 1.0	−1.6 ± 0.6	0.66
Total femur, T-score	−0.9 ± 1.0	−0.9 ± 1.0	−1.0 ± 0.7	0.91

[a] One-way ANOVA for continuous variables; Chi-square test for categorical variables.
RE: resistance exercise; AE: aerobic exercise; CON: control.

2.2.1. Bone and body composition

BMD was measured using dual-energy X-ray absorptiometry (DXA) (QDR 4500A, Hologic, Bedford, MA) at the proximal femur on the nondominant side using standard protocols. To minimise interobserver variation, the same investigator carried out all analyses. Bone phantoms were scanned daily, and coefficients of variation (CV) were verified before and during the experimental period to ensure assessment reliability.

Total body scans were taken using the same DXA instrument. All scans were performed by the same technician using standard procedures, as described in the Hologic user's manual. Scans were analysed for total lean mass, fat free mass and percent body fat mass. Fat free mass consists of lean mass and bone mineral mass. Lean mass (i.e., bone-free fat free mass) was included into the analysis as a surrogate measure of muscle mass. Because the exercise protocols were designed to improve bone tissue, fat free mass was included as a comprehensive measure expressing the pooled change in bone and lean mass.

To test the precision of our DXA scanner, repeated scans were performed on 15 healthy older adults. Each individual underwent three consecutive total-body and hip scans with repositioning. The CV (standard deviation/mean) for repeated measurements was 0.8% for total body BMD, 0.9% for femoral neck BMD and 1.1% for total femur BMD. CV-values for percent body fat, fat free mass, and lean body mass were 3.1%, 2.8%, and 1.1%, respectively.

Height and body mass were recorded using a portable stadiometer and balance weighing scales, respectively. Body mass index (BMI) was calculated using the standard formula: mass (kg)/height^2 (m).

2.2.2. Muscular strength

The dynamic concentric muscle strength of the lower extremities, namely the knee flexion (KF) and extension (KE) muscle groups, was measured on an isokinetic dynamometer (Biodex System 4 Pro; Biodex, Shirley, NY). Strength measurements were carried out, in accordance with the manufacturer's instructions for KE/KF, at two angular velocities, 60°/s (1.05 rad s^{-1}) and 180°/s (3.14 rad s^{-1}). Each participant, after familiarisation with the machine, performed five maximal efforts at 180°/s and three at 60°/s with two minutes of rest between tests. The dynamometer angle reading was calibrated to the anatomic joint angle measured by a goniometer, and gravity corrections to torque were based on leg weight at 0° and calculated later by the equipment software. Prior to testing, subjects performed a five minute warm-up on a bicycle ergometer (Bike-Max; Tectrix, Irvine, CA) at 45–60 W. During the test, participants were verbally encouraged to exert maximal muscular force. Peak torque, represented as a percentage normalised to body weight, was used for the statistical analyses.

2.2.3. Balance and mobility performance measures

Each subject performed two balance tests. Mobility/dynamic balance was assessed using the 8-foot Up and Go Test (8-ft UG test) (Rikli and Jones, 1999) and static balance was measured using the one-leg stance (OLS) (Bohannon, 1994). Before starting the tests, participants remained seated and rested for five minutes. In the 8-ft UG test, the score corresponded to the shortest time to rise from a seated position, walk 2.44 m (8 ft), turn, and return to the seated position, measured to the nearest 1/10th s. The OLS test involved standing upright as still as possible in a unassisted unipedal stance (on the nondominant leg) on a 40–60 cm force platform (Force Plate AM 4060–15; Bertec, Columbus, OH) with eyes open, head erect, and arms by the side of the trunk.

The OLS was timed in seconds from the time one foot was lifted from the floor to when it touched the ground or the standing leg. A longer time indicated better balance; the maximum time was set at 45 s. Two attempts were allowed, with one minute of rest between, and the best performance was used for force-platform-based analysis.

The signals from the force platform were sampled at 500 Hz. We used a personal computer to collect the data with the customised AcqKnowledge-based software (AcqKnowledge 3.9.1; Biopac, Goleta, CA). The data analysis was performed using MATLAB software (MATLAB 7.0; MathWorks, Natick, MA). Data from horizontal forces (Fy and Fx) and centre of pressure (COP) time-series were low-pass filtered with a zero-lag, fourth-order Butterworth filter with a cut-off frequency of 10 Hz.

The outcome variables were anterior–posterior (AP) and medial–lateral (ML) mean velocity (cm s^{-1}) of the COP; the elliptical area (EA) was calculated using the equation: $\sqrt{2}\sigma y \times \sqrt{2}\sigma x$. Mean velocity was determined by dividing the total distance along the signal trajectory by the total recording time.

2.2.4. Blood sampling and serum measurements

Fasting venous blood samples were drawn between 7.30 and 9.30 a.m. After collection, blood samples were collected in tubes containing EDTA, and serum samples were clotted at room temperature for 90 min and were then centrifuged for 10 min at 1000 × *g*. Samples were aliquoted and stored at −80 °C until analysis. OPG and RANKL were determined by a commercial sandwich enzyme-linked immunosorbent assay (ELISA) according to the protocol of the manufacturer (Immunodiagnostic Systems Ltd, Boldon, UK and Cusabio Biotech, China, respectively). The same serum samples used for RANKL measurements were used for OPG measurements, and the assay was performed blind to the subject group. The detection limit was 0.140 pmol/L for the OPG assay and <31.2 pg/mL for RANKL assay, with an intra- and inter-assay CV of <10%.

2.2.5. Lifestyle behaviours and clinical status

A baseline self-administered questionnaire to assess the impact of present and past lifestyle choices was completed by interview to avoid misinterpretation of items and/or skipping of questions. The questionnaire included information regarding education; marital status; fall and fracture history; medical history; current medical conditions; medication use; current and past PA; age at menarche; menopause status; current and previous use of hormone replacement therapy; past dietary habits, including calcium intake; and current and past smoking.

2.2.6. Daily PA

The Actigraph GT1M accelerometer (Manufacturing Technology, Fort Walton Beach, FL) was used as an objective measure of daily PA, using a 15 second measurement interval (epoch). All participants agreed to wear an accelerometer for seven consecutive days and were instructed to wear the device over their right hip using an adjustable nylon belt. Exceptions included time spent sleeping and showering. Participants were asked to maintain usual activities. For data to be included in the analyses, participants were required to wear the accelerometer for at least four of the seven days. For both test periods (pre- and post-trainings), four files were corrupt, and six files had only two valid days. Those ten participants were contacted and agreed to wear the accelerometer again for seven days (one week later than the rest of the group). In total, pre- and post-training data from all participants were included in the analysis (90 files with seven valid days, 7 files with 6 valid days, and 11 files with 5 valid days).

The cut point was set at counts per minute ≥1041 (moderate to vigorous PA [MVPA]) which corresponded to a mean VO_2 of 13 mL kg^{-1} min^{-1}, based on the counts associated with a reference activity, which was walking at 3.2 km/h (Copeland and Esliger, 2009). The average daily moderate to vigorous PA, number of steps, and daily activity counts per minute (cpm) were analysed.

2.2.7. Nutritional assessment

Nutritional status was assessed using 4-day diet records over three weekdays and one weekend day. To ensure standardisation of the

dietary records, a dietician gave individual instruction to the subjects concerning how to fill out the diet records and assess food serving sizes. Diet records were analysed using Food Processor Plus® (ESHA Research, Salem, OR), which uses the table of food components from the U.S. Department of Agriculture. Some traditional Portuguese dishes were added based on the table of Portuguese food composition. Total caloric, protein, calcium, phosphorous, vitamin D, and caffeine intakes were compared between the RE, AE and CON groups.

2.2.8. Lactose persistence status

The lactose persistence mutation, C/T −13910, was genotyped by direct sequencing. A 359-bp fragment containing all mentioned mutations and located in the intron 13 of the *MCM6* gene was amplified using primers 5′-GCAGGGCTCAAAGAACAATC-3′ (forward) and 5′-TGTTGCATGTTTTTAATCTTTGG-3′ (reverse). PCRs reactions contained 0.5 μM of each primer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 750 mM Tris–HCI (pH 8.8 at 25 °C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, 1.5 mM $MgCl_2$ and 1 U Taq polymerase. The PCR profile consisted of the following: 94 °C for five minutes, 35 cycles of 94 °C for one minute, 58 °C for one minute and 72 °C for one minute, followed by 20 min of extension at 72 °C.

Sequencing reactions were carried out using the ABI Big Dye v3.1 Ready Reaction Kit and using the protocol specified by the manufacturer (Applied Biosystems, Foster City, CA). Products were run on an ABI PRISM 3130×1 sequencer and analysed in the ABI PRISM 3130×1 Genetic Analyser software (Applied Biosystems). The resulting chromatograms were inspected for the presence/absence of lactase mutations using the MEGA4.0 software (www.megasoftware.net) (Tamura et al., 2007).

DNA was obtained from buccal swabs using standard extraction methods.

2.3. Exercise protocol

2.3.1. Aerobic exercise

The AE group completed a 32-week endurance exercise training programme consisting of three sessions per week, with at least one day of rest between sessions. Each session lasted approximately 60 min, and all sessions were accompanied by appropriate music relevant to the required activity and participants' age. The exercise training consisted of stretching and warm-up exercises (10–15 min), dynamic aerobic activities (35–40 min) involving stepping, skipping, graded walking, jogging, dancing, aerobics and step choreographies, and cool-down/relaxation exercises (10 min). During the first six weeks, moderate intensity strength exercises were performed concentrically and eccentrically for approximately ten minutes for the hip flexors, extensors, and abductors; knee flexors and extensors; and ankle dorsiflexors and plantar flexors using body weight to ensure proper muscular resistance and to sustain the increments in training intensity. The initial exercise intensity was set at 50% to 60% of the subjects' heart rate reserve for the first two months; the target heart rate during exercise was continuously monitored by heart rate monitors (Polar Vantage XL, Polar Electro Inc., Port Washington, NY), and the rate of perceived exertion was assessed using Borg's 10-point psychometric scale (Borg et al., 1987). Exercise intensity was gradually increased from 65% to 85% of the heart rate reserve as adapted to the individual. Each session was led by three physical education instructors who specialised in PA for older adults and was supervised by the researchers.

2.3.2. Resistance exercise

RE sessions were performed three times per week on nonconsecutive days, with each session lasting approximately 60 min over a period of 32 weeks. All training sessions were conducted at University of Porto, Faculty of Sport Facilities and were supervised by three research assistants who were responsible for warm-up, cool down, and stretching exercises; the monitoring of correct lifting form; the appropriate amount of exercise and rest intervals; the maintenance of daily exercise logs; and the progression of the exercises. Subjects were also encouraged to exercise with a training partner to provide additional motivation. Each training session involved the following: (1) a standardised warm-up period (8–10 min) on a bicycle ergometer (Bike-Max; Tectrix, Irvine, CA) and/or rowing ergometer (Concept II, Morrisville, VR) at low intensity and some stretching exercises; (2) specific resistance training period (30–40 min); and (3) a cool-down period (5–10 min) that included walking and stretching exercises. The RE protocol aimed to develop muscle mass and strength in the following muscle groups: (1) quadriceps (leg press and leg extension), (2) hamstrings (seated leg curl), (3) gluteal (hip abduction), (4) trunk and arms (double chest press, lateral raise and overhead press), and (5) abdominal wall (abdominal machine). Subjects exercised on variable resistance machines (Nautilus Sports/Medical Industries, Independence, VA). To minimise fatigue, the exercises for the upper/lower parts of the body were performed in a non-consecutive way, with a rest period of approximately two minutes between each set. Each repetition lasted three to six seconds, involving a period of at least two minutes between the two sets of 10–12 repetitions at 60–70% of 1RM. Training intensity was gradually increased during the first four weeks. Participants underwent a 2-week familiarisation period with the equipment and the exercises. The intensity of the training stimulus was initially set at 50% to 60% of one-repetition maximum (1RM), as determined at week 2, with a work range of two sets of 10 to 15 repetitions. Subjects then progressed from 75% to 80% of 1RM at a work range of six to eight repetitions (two sets) and remained at this level until the end of the programme. Training was continuously monitored by heart rate monitors (Polar Vantage XL, Polar Electro Inc., Port Washington, NY) and ratings of perceived exertion (Borg's 10-point psychometric scale) (Borg et al., 1987). 1RM tests were performed every two weeks for the first month and then every four weeks until the end of the programme. Between these tests, the load was increased for those subjects who were able to easily complete 12 or more repetitions for both sets.

Exercise compliance was defined as the number of exercise sessions reported divided by the number of maximum exercise sessions possible.

2.4. Statistical analysis

All statistical analyses were performed using PASW Statistics (version 18; SPSS, Inc., Chicago, IL) for Windows with a significance level of 0.05. Data were checked for distribution, and the means ± SD were calculated. Primary outcomes were changes from baseline in response to both 8-month interventions in balance, muscle strength, BMD and serum level of OPG and RANKL. Secondary outcomes included 8-month changes from baseline in dietary intake, daily PA, body composition (BMI, waist circumference, fat, fat-free mass, and lean mass), and the presence or absence of the lactase mutations. The results were analysed on an intention-to-treat basis, and missing data due to lack of follow-up (the method assumed data were missing at random) were replaced using the process of multiple imputation. This method has been adapted to the analysis of longitudinal data (Mazumdar et al., 1999). Potential differences amongst groups in baseline measurements were evaluated using one-way analysis of variance (ANOVA). Chi-squared tests were used for between-group comparisons of categorical variables at baseline. Pearson correlations were used to determine the relationship of potential confounding variables (e.g., lactase persistence, dietary intake, PA change, and fat mass change) with primary outcomes. Such confounding variables were then entered as covariates in the analysis of variance model as indicated. The delta percentage was calculated with the

standard formula: % change = [(posttest score − pretest score) / pretest score] × 100.

A two-way (group and time) factorial ANOVA, with repeated measures on one factor (time), was performed for differences in main effects and time by group interactions for each dependent variable. Main effects were considered when interactions were not significant. When significant interactions were found, Bonferroni post hoc tests were used to determine significant differences amongst mean values.

A power analysis based on a formulation of 75% power, an effect size of 0.5 for overall muscle strength, balance and BMD from previous studies, and a significance level of 0.05 for a one-tailed test deemed that a sample of 23 per group was sufficient to address the research questions.

3. Results

3.1. Recruitment

Of the 71 women aged 69.0 ± 5.3 (range 61–83) who underwent the initial assessment and randomisation, 44 were randomised to the following three groups: RE, n = 15; AE, n = 19; and CON, n = 20. One participant discontinued the intervention because of surgery, and five participants discontinued due to medical issues unrelated to the intervention. Two participants left the study due to unwillingness to participate, three due to loss of interest and six to personal reasons. As expected, dropout rates were higher in the exercise groups (8 RE, 5 AE) than the CON group (n = 4) because of the time commitment. However, no differences ($p = 0.315$) in dropout rates were observed between groups. Fig. 1 shows the number of participants at each stage of the study.

3.2. Subject characteristics

Demographics and descriptive parameters of all groups are listed in Table 1. Of the participants, 73% of the participants were overweight, most of them had hypertension, and a small proportion had a history of cigarette smoking. On average, participants obtained 85 min of MVPA per day. The molecularly defined prevalence of lactase persistence (TT/TC genotypes) was similar for all groups. The prevalence of the TT and CT genotypes of the 13910 C/T polymorphism was 25.0% and 28.6%, respectively. There were no significant group differences in any baseline characteristic.

3.3. Compliance with intervention and adverse events

One-hundred percent compliance to the exercise sessions was set at 96 training sessions. Excluding dropouts, mean compliance to the RE sessions was 78.4% (61.6–95.9%), and for AE training, the mean compliance was 77.7% (64.2–96.8%). There were no exercise- or assessment-related (pre- and posttraining) adverse events.

In comparison to individuals who completed the trial, those who failed to provide follow-up data had no significant differences in any baseline measurements, including age, body weight, daily MVPA levels, strength, balance, or BMD.

3.4. Dietary intake

Total energy intake was similar amongst the groups at baseline and during the period of intervention. Energy intake was 1473 ± 318 kcal/day at baseline and 1499 ± 302 at eight months ($p > 0.05$ for all group changes). No group differences were apparent in baseline values (Table 1) or change in dietary protein, phosphorus, caffeine,

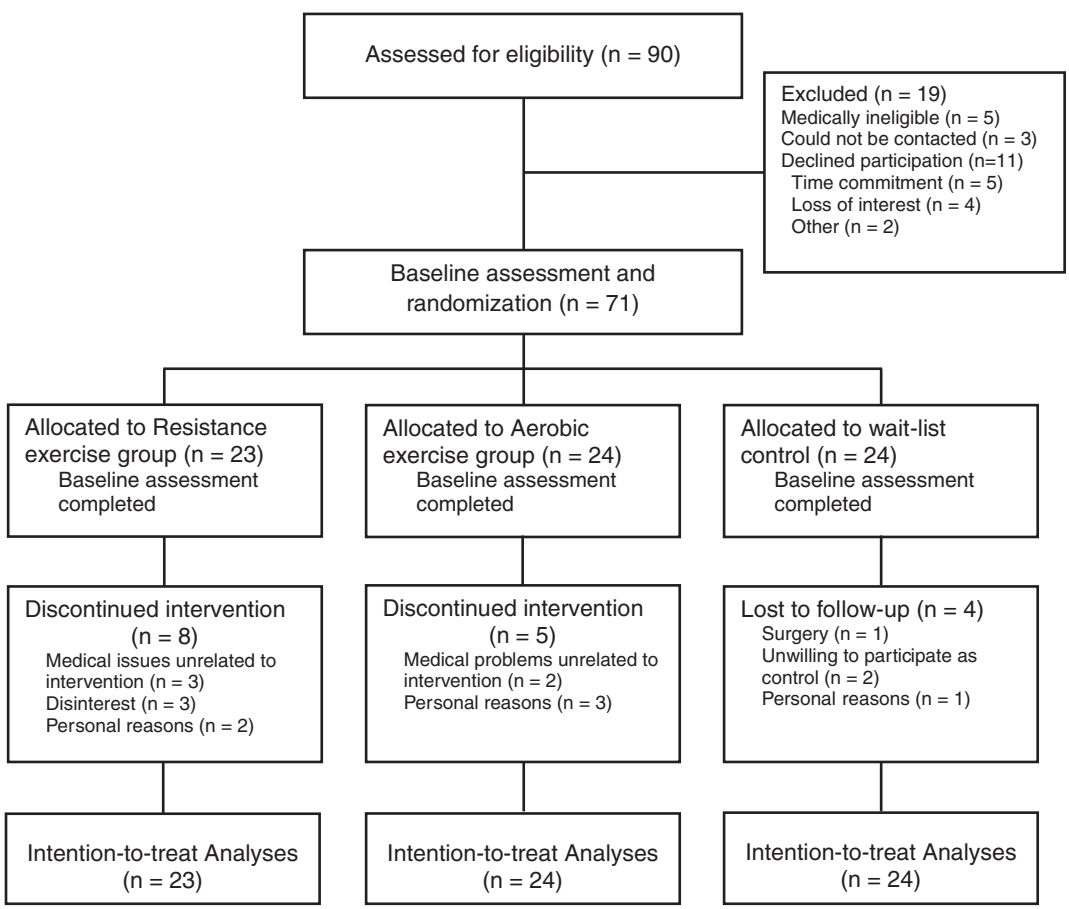

Fig. 1. Flow of participants through the study.

calcium, and vitamin D intake in response to the interventions (data not shown). No significant difference in mean daily total calcium intake derived from milk and dairy products was evident amongst those with and without lactase persistence within each group.

3.5. Daily PA

No significant changes in MVPA level were observed at eight months. There was no significant interactive ($p=0.417$) or main effect of group ($p=0.214$) and time ($p=0.171$) on changes in PA. Changes in MVPA were not related to changes in the primary outcomes.

3.6. Changes in body composition

No significant interaction occurred for BMI or waist circumference in response to exercise intervention. There was a main effect of time ($p=0.039$) on waist circumference. Interactions were observed for lean mass ($p=0.026$), fat free mass ($p=0.030$), and percent fat mass ($p=0.028$; Table 2), such that only the RE group significantly increased lean and fat free mass and decreased percentage fat mass, whereas no significant changes were observed for the AE and CON groups.

3.7. Changes in balance and muscle strength

No significant difference between groups for the variables was apparent at baseline. There were significant interactions between group and time on all measurements of balance and strength (Table 3). Accordingly, both RE and AE groups improved the time to perform both balance tests, and a significant difference for post-training results was evident between exercise intervention groups and the CON group for EA and velocity values for ML-direction. However, only the AE group significantly decreased the mean velocity of the COP displacement for AP-direction. A significant decrease in 8 ft UG performance was observed for the control group; the trend indicated a decline in all balance and strength variables. Regarding muscle strength, only the RE group significantly increased their maximal KE and KF torques at both speeds.

3.8. Changes in BMD

At baseline, there were no significant differences amongst the groups in BMD at any site measured (Table 4). There were significant interactions between group and time on BMD at the trochanter ($p=0.005$) and total hip ($p=0.034$). Accordingly, the RE group significantly increased BMD by 2.9% (0.020 g/cm^2) at the trochanter and 1.5% (0.013 g/cm^2) at the total hip (Fig. 2). No significant changes in BMD were observed for the AE and control groups ($p>0.05$). There was no significant interaction or main effects of group and time on serum OPG and RANKL levels or the OPG/RANKL ratio (all $p>0.05$; Table 4, Fig. 2).

In the collective sample ($n=71$), changes in caffeine intake were significantly related to changes in trochanter BMD ($r=-0.26$, $p=0.045$). Nevertheless, the interaction of trochanter BMD remained significant ($p=0.003$) after controlling for changes in caffeine intake. Changes in percent fat mass were significantly related to changes in intertrochanteric region BMD ($r=0.27$, $p=0.035$). The lack of a significant interaction between group and time, and the main effects on the intertrochanteric region remained unchanged after adjusting for change in total percent fat mass.

4. Discussion

Age-related functional changes, including reduced balance, gait ability and muscle strength, have been consistently related to fall risk. Given that low BMD along with the above-mentioned functional declines combine to make older adults, especially women, much more prone to bone fractures, it is reasonable to determine whether BMD, balance and strength might significantly increase after long-term exercise training and what type of exercise could induce the most pronounced effects in elderly women. Moreover, the regulation of osteoclastic activity is critical for understanding bone changes induced by exercise (mechanical load). OPG and RANKL have been shown to be important regulators of osteoclastogenesis, although few comprehensive efforts have been made to characterise the effects of long-term exercise on serum expression of both cytokines. Overall, data from the present study have shown that RE increases the BMD at the trochanter and total hip, balance and strength and that these effects are more pronounced than after AE in older women. No changes were observed in OPG and RANKL after eight months of exercise.

The use of exercise as a possible prevention strategy for prevention of fractures in elderly people has previously been hypothesised (Vogel et al., 2009). However, results have been discordant, depending on the type, intensity, duration of exercise, and on participants' age and functional status. To detect relevant biomechanical changes in postural stability, force platform-based measures were obtained during the OLS test. Although the effectiveness and validity of force platforms to assess postural balance in older people have been established (Pajala et al., 2008), few studies have documented the results of force platform balance test data. The present work confirmed that both resistance and aerobic exercise resulted in increased static and dynamic balance. For instance, favourable changes in postural sway, including decreased EA and slower velocity of COP displacement have previously been reported after exercise training (Messier et al., 2000). In addition, instability and age are expected to increase the EA and the velocity of COP trajectories (Abrahamova and Hlavacka, 2008; Latash et al., 2003). These results are somewhat surprising, as several studies have failed to support exercise-related benefits on balance, although these studies generally assess balance after single interventions without a specific balance/proprioceptive-related training component (Manini et al., 2007; Henwood and Taaffe, 2006). Furthermore, we observed that only RE

Table 2
Pre- and post-training values for body composition variables.

Variable	Resistance exercise group		Aerobic exercise group		Control group		p (group)	p (time)	p (interaction)
	Pre-training	Post-training	Pre-training	Post-training	Pre-training	Post-training			
BMI, kg/m^2	28.8 ± 4.6	28.2 ± 3.9	27.5 ± 3.8	27.5 ± 3.3	28.1 ± 3.5	27.3 ± 2.0	0.648	0.107	0.377
WC, cm	93.0 ± 10.5	91.2 ± 8.2	89.1 ± 9.5	86.7 ± 6.8	91.4 ± 8.7	90.8 ± 10.7	0.293	0.039	0.580
Lean mass, kg	41.8 ± 8.6	44.6 ± 8.6[a,b,c]	37.3 ± 5.2	37.2 ± 5.2	39.4 ± 5.0	38.2 ± 3.2	0.006	0.386	0.026
Fat free mass, kg	43.6 ± 8.9	46.6 ± 9.1[a,b,c]	39.0 ± 5.4	38.9 ± 5.6	41.1 ± 5.1	39.9 ± 3.4	0.006	0.378	0.030
Fat mass, %	38.8 ± 4.4	35.2 ± 5.5[a]	39.2 ± 4.5	38.4 ± 3.8	38.4 ± 4.6	37.8 ± 3.7	0.402	0.001	0.028

BMI: body mass index; WC: waist circumference.
[a] Indicates a significant intra-group difference, $p<0.05$.
[b] Indicates a significant difference from AE Group at post test, $p<0.05$.
[c] Indicates a significant difference from CON Group at post test, $p<0.05$.

Table 3
Pre- and post-training values for muscle strength, dynamic and static balance.

Variable	Resistance exercise group		Aerobic exercise group		Control group		p (group)	p (time)	p (interaction)
	Pre-training	Post-training	Pre-training	Post-training	Pre-training	Post-training			
8 ft UG, s	5.5 ± 0.5	4.9 ± 0.3[a]	5.9 ± 0.9	5.1 ± 0.6[a]	6.0 ± 0.8	6.3 ± 1.2[a]	<0.001	<0.001	<0.001
OLS, s	26.3 ± 13.2	31.7 ± 12.8	28.8 ± 14.9	32.9 ± 9.5[b]	26.9 ± 16.2	22.3 ± 13.6	0.221	0.327	0.028
EA, cm^2	7.4 ± 4.8	3.3 ± 1.0[a,b]	7.2 ± 4.3	3.3 ± 1.3[a,b]	7.2 ± 4.4	7.4 ± 4.4	0.063	<0.001	0.001
AP velocity, cm s^{-1}	4.0 ± 1.1	3.5 ± 0.8	3.7 ± 1.1	3.0 ± 0.8[a,b]	4.0 ± 1.1	4.4 ± 1.5	0.025	0.027	0.003
ML velocity, cm s^{-1}	4.7 ± 2.1	3.3 ± 0.8[a,b]	4.3 ± 1.9	2.9 ± 0.8[a,b]	4.8 ± 2.1	4.6 ± 2.4	0.085	<0.001	0.041
KE PT/BW 180°/s, %	76.2 ± 16.0	90.5 ± 15.1[a]	84.5 ± 21.5	81.2 ± 24.9	81.3 ± 18.6	79.1 ± 19.3	0.819	0.244	0.013
KF PT/BW 180°/s, %	50.5 ± 18.3	61.6 ± 14.9[a,b]	47.2 ± 12.8	51.7 ± 11.8	50.6 ± 15.0	49.9 ± 11.1	0.211	0.010	0.047
KE PT/BW 60°/s, %	123.0 ± 29.8	140.7 ± 31.1[a]	137.7 ± 36.5	132.5 ± 28.2	134.4 ± 27.3	129.6 ± 28.6	0.915	0.424	0.010
KF PT/BW 60°/s, %	74.6 ± 23.4	94.4 ± 24.5[a,b]	71.8 ± 19.0	77.1 ± 19.3	68.6 ± 20.0	66.6 ± 20.2	0.026	0.003	0.003

8 ft UG: 8-foot Up and Go Test; OLS: one leg stance; EA: elliptical area; AP: anterior–posterior; ML: medial–lateral; KE: knee extension; KF: knee flexion; PT: peak torque; BW: body weight.
[a] Indicates a significant intra-group difference, $p<0.05$.
[b] Indicates a significant difference from CON Group at post test, $p<0.05$.

had a significant effect on maximal knee extension and flexion strength. Although there is consensus that older adults can substantially increase their strength and power after RE (Chodzko-Zajko et al., 2009), the effectiveness of AE is questionable, with a number of studies showing significant improvements (Misic et al., 2009; Nalbant et al., 2009) and others reporting no evidence of muscle strength or power increase (Haykowsky et al., 2005; Tarpenning et al., 2006). Together, these findings reinforce the notion that exercise training has the potential to reduce fall risk.

The mechanical loading of bone through exercise has been investigated thoroughly for its potential to positively alter structural variables, including bone mass (Bailey and Brooke-Wavell, 2008). This change in bone as a result of exercise has been attributed to strain (defined as the fractional change in the dimension of a bone in response to a changing load) (Kohrt et al., 2004), which represents the key intermediate variable, and to its effect on cells by directly changing their dimensions or indirectly impacting intralacunar pressure, shear stresses, or charged fluid flow (Lanyon, 1996). In addition, accumulating evidence suggests that high strain rates and unusual strain distributions are positively related to osteogenic response (Bailey and Brooke-Wavell, 2008). In the present study, the RE-based intervention significantly increased BMD at the trochanter and total hip. Furthermore, no significant changes resulted after eight months of AE and a non-significant trend towards diminished bone density in the control group was observed. Together, these observations suggest that aerobic training protocols, which include concentric and eccentric muscle actions and loading impacts although at a lower intensity than with RE, produce modest effects on BMD in older women. In fact, despite being an important training mode, especially for the induction of cardiovascular and metabolic changes (Chodzko-Zajko et al., 2009), previous studies using aerobic training protocols have reported conflicting results regarding BMD. Although Silverman et al. (2009) found a significant improvement of 2% at the femoral neck in postmenopausal women after a 24-week walking programme, a number of studies have shown limited bone density improvements in postmenopausal women after AE (Palombaro, 2005; Martyn-St James and Carroll, 2008). One possible explanation is that aerobic protocols based only on walking activities, which lack lateral and twisting movements, do not represent a unique stimulus to bone. Conversely, our aerobic protocol included more diverse activities, such as jogging, skipping, step climbing/descending, dancing, aerobics and step choreographies. In fact, the data from Kohrt et al. (1997) demonstrated that an exercise programme including walking, jogging and stair climbing resulted in significant increases in BMD of the whole body, lumbar spine, femoral neck, and Ward's triangle. Conversely, our results suggest that the strain levels induced by the present AE were not sufficient to improve bone mass.

Our results on the RE-induced elevation in BMD are consistent with prior studies that have similarly reported the effectiveness of exercise in promoting an osteogenic response in elderly adults (Ryan et al., 2004; Bemben and Bemben, 2010). However, there is still some controversy regarding its osteogenic potential in older adults. Previous studies have described a lack of significant alterations in BMD at the proximal femur or lumbar spine after progressive resistance exercise programmes (Rhodes et al., 2000; Stengel et al., 2005). To date, several studies have focused on the impact of resistance exercise interventions on bone mass in premenopausal women (Martyn-St James and Carroll, 2006). Nevertheless, some of them have also reported a lack of BMD response to resistance training (Singh et al., 2009; Nakata et al., 2008). Given the heterogeneity of women's responses at different ages (likely due to a deterioration of the ability of older bone cells to perceive these physical signals or a failure of their capacity to respond) and the fact that oestrogen withdrawal is associated with increased remodelling intensity (Lanyon and Skerry, 2001), inconsistent results between studies amongst pre-, postmenopausal and older women can be anticipated. However, whilst strategies to increase bone mass in young premenopausal women can involve high-impact exercises, such as vertical

Table 4
Eight-month changes for BMD, OPG, RANKL and OPG/RANKL.

Variable	Resistance exercise group		Aerobic exercise group		Control group		p (group)	p (time)	p (interaction)
	Pre-training	Post-training	Pre-training	Post-training	Pre-training	Post-training			
Femoral neck, g/cm^2	0.684 ± 0.082	0.676 ± 0.090	0.657 ± 0.105	0.660 ± 0.111	0.678 ± 0.056	0.676 ± 0.065	0.719	0.641	0.553
Troch, g/cm^2	0.646 ± 0.095	0.666 ± 0.106[a]	0.638 ± 0.099	0.641 ± 0.098	0.628 ± 0.038	0.621 ± 0.046	0.448	0.012	0.005
Inter, g/cm^2	1.035 ± 0.168	1.047 ± 0.164	1.022 ± 0.141	1.020 ± 0.142	0.990 ± 0.085	0.980 ± 0.113	0.368	0.334	0.343
Total hip, g/cm^2	0.859 ± 0.124	0.873 ± 0.132[a]	0.848 ± 0.125	0.849 ± 0.124	0.831 ± 0.065	0.824 ± 0.082	0.468	0.006	0.034
OPG, pmol/L	5.46 ± 1.24	5.42 ± 1.00	7.58 ± 3.91	7.21 ± 4.43	10.10 ± 6.24	9.06 ± 4.94	0.062	0.117	0.420
RANKL, pmol/L	171.56 ± 86.26	152.73 ± 63.76	180.78 ± 85.77	188.32 ± 89.99	157.49 ± 63.25	171.11 ± 64.80	0.661	0.880	0.052
OPG/RANKL	0.037 ± 0.012	0.039 ± 0.010	0.056 ± 0.044	0.048 ± 0.036	0.067 ± 0.040	0.058 ± 0.036	0.222	0.054	0.185

BMD = bone mineral density, Troch = trochanter, Inter = intertrochanteric region.
[a] Indicates a significant intra-group difference, $p<0.05$.

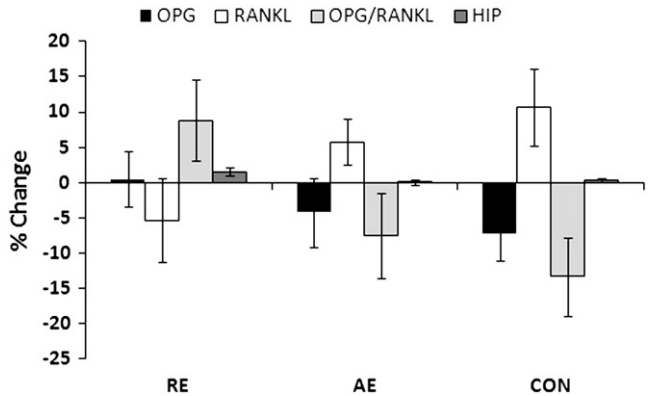

Fig. 2. Percentage of changes from baseline in serum OPG, RANKL an OPG/RANKL ratio, and bone mineral density of the proximal femur in response to exercise or placebo (control) over 8 months. Values are mean ± SEM. RE: resistance exercise; AE: aerobic exercise; COM: control group.

jumping, exercises that introduce high strain levels to the skeleton are difficult to perform with advancing age due to the high risk of traumatic fractures, stress injuries and arthritic complications. In such circumstances, the optimal exercise prescription for older people should meet other paramount needs, including being feasible, safe, acceptable, and cost-effective. The positive effect observed at the total femur and trochanter is probably related, at least partially, with the inclusion of movements, such as hip abduction that stimulates the gluteus medius and minimus, as both insert on the greater trochanter of the femur, and are also assisted by the lateral rotator group which inserts on or near the greater trochanter of the femur. The hip flexors (iliacus and psoas major) play an important role in the leg press exercise, and may also have an osteogenic effect at the femur as it connects to the lesser trochanter.

In this study, serum OPG level, serum RANKL levels and the OPG/RANKL ratio did not change significantly after an 8-month RE and AE training programme. Previous data have shown that mechanical stimulation (i.e., dynamic flow-induced shear stress) induced in vitro inhibits osteoclastogenesis through an upregulation of OPG and a downregulation of RANKL (Kim et al., 2006). Saunders et al. (2006) also found that mechanical stimulation via substrate deformation significantly increases soluble OPG levels by osteoblastic cells. Despite the above-mentioned results from in vitro studies and other promising results of long-distance running effects on BMD via the OPG/sRANKL system (Ziegler et al., 2005), there are no data available in the literature concerning the effectiveness of common exercise modalities, such as RE and AE on serum OPG and RANKL levels amongst older adults. Our results show that eight months of RE and AE training do not elicit significant changes in either biomarkers. Although it is well known that OPG blocks the differentiation of pre-osteoclastic cells into osteoclasts by inhibiting the binding of RANK to RANKL and, consequently, reduces osteoclastic bone resorption (Boyle et al., 2003), not all mechanical stimulation studies have shown an increase in OPG with osteoblast stimulation (Liegibel et al., 2002). Moreover, Esen et al. (2009) reported no significant changes in OPG levels after a 10-week walking programme in middle-aged men. An increased expression of RANKL may be involved in the excessive bone resorption observed in osteoporosis, thus a down-regulation of RANKL should prevent bone loss. There is evidence from in vitro studies that mechanical load may induce down-regulation of RANKL (Rubin et al., 2003; Rubin et al., 2000; Lau et al., 2010). In contrast to these studies, our findings showed that exercise did not favourably affect RANKL levels. One study observed exercise-induced RANKL changes, reporting that a 10-week high intensity walking programme significantly reduced RANKL in middle-aged men (Esen et al., 2009). Conversely, in a previous study using human cell lines, mechanical stimulation did not affect RANKL (Saunders et al., 2006). The reasons for this dissimilarity in results, apart from the disparity in setting (in vitro and in vivo studies), may stem from the differences in mechanical stimulation mode, intervention length, and physiological factors, such as cyclic variations, age and gender.

A major limitation of this study is the lack of concurrent measures of the magnitude of the applied loads based on calculations of the ground reaction forces (GRF). Moreover, we used an accelerometer cut point of 1041 cpm which is substantially lower than the cut point of 1952 cpm that is typically used for moderate activity in younger adults (Freedson et al., 1998). Indeed, using the former cut point the mean MVPA decreases to 32 min. Although the 1041 cpm may be more appropriate to our sample age, this cut point was obtained from a small sample of older adults and no vigorous activity was included in the calibration protocol, which may overestimate the time spent in moderate activity. The mechanical competence of bone is a function not only of its intrinsic material properties (mass, density and stiffness), but also of its structural properties (size, shape and geometry). DXA is the method most commonly used to measure BMD area (g/cm^2) because of its speed, precision, low radiation exposure and availability of reference data (Watts, 2004); however, this two-dimensional skeletal outcome represents only one part of overall bone strength. Finally, our sample size may have been too small to detect significant changes in all variables.

A key strength of our study is the novel data it provides regarding the relationship between exercise training modes and OPG levels in this particular population. Moreover, this study has considered the possible influence of critical confounding variables, such as daily PA levels objectively measured by accelerometers, nutrition and lactose persistence (genetically defined by the C/T −13910 genotype). Lactase deficiency has been associated with BMD (Obermayer-Pietsch et al., 2004) and reduced intake of calcium (Bacsi et al., 2009) and could represent a genetic risk factor for bone fractures for older adults (Enattah et al., 2005). However, no associations were found between lactase deficiency status and reduced BMD or calcium intake and BMD changes after training.

In conclusion, the results indicate that 8-month RE, but not AE, can induce significant bone adaptation in older women without significantly affecting OPG and RANKL levels. We have also demonstrated that both exercise training regimens elicited significant gains in balance. The results presented here suggest that higher workloads may be necessary in AE programmes to increase bone mass. Although these findings provide some clue into the potential for exercise to reduce fracture risk in community-dwelling older women, additional data are needed to validate and build upon our findings using additional outcome measures.

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Paper IV

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Response of bone mineral density, inflammatory cytokines, and biochemical bone markers to a 32-week combined loading exercise programme in older men and women

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Abstract

The aim of this study was to examine the effects of 8-month exercise training on balance, bone mineral density (BMD), biochemical marker of bone metabolism, inflammatory cytokines and C- reactive protein (CRP) concentrations in older men and women in a nonrandomized controlled trial. Forty-seven healthy older adults (61-84 years) participated in a exercise training intervention that included resistance exercise training (2 days/week) plus a multicomponent weight-bearing impact exercise training (1 day/week) for 32 weeks. Outcome measures included lumbar spine and proximal femoral BMD (by dual X-ray absorptiometry), dynamic balance, serum osteocalcin (OC), C-terminal telopeptide of Type I collagen (CTX), osteoprotegerin (OPG), receptor activator of nuclear factor kappa B ligand (RANKL), high sensitive (hs)-CRP, and the inflammatory cytokines [interleukin (IL)-6, tumor necrosis factor (TNF-α), and interferon (IFN)-γ]. Potential confounding variables included body composition data, dietary intake (using 4-day diet records), and accelerometer-based daily physical activity. No significant changes were observed in secondary outcomes. After 32 weeks, both men and women significantly increased dynamic balance, trochanter, intertrochanter, total hip, and lumbar spine BMD, while OC, CTX, OPG and RANKL remained unchanged. In addition, hs-CRP and IFN-γ significantly decreased, while there were no significant group or time effects in TNF-α. A significant decreased in IL-6 was observed only in men. The change in TNF-α was negatively correlated with the change in lumbar spine BMD ($p<0.05$).

Therefore, despite the lack of significant bone metabolism alterations with exercise training, our combined impact protocol is recommended to decrease inflammation, and to increase balance and BMD in healthy older adults, thus reinforcing the preventive and therapeutic role of exercise to the age-related inflammation, balance and bone loss.

Key Words: Bone mass; elderly; mechanical load; inflammation; biomarkers

1. Introduction

Bone is a unique tissue, able to react to local mechanical loading which optimizes its structure, namely bone mass, geometry, and material properties. Although bone's ability to adapt to physical forces was recognized long time ago, aging skeleton's response to load is affected by several age-related changes which contribute to an attenuation of the adaptive response [1]. Previous observations of bone's response to exercise have produced inconsistent results with respect to changes in bone density in older adults [2-5]. These discrepancies may be attributed to differences in participants' age (i.e. postmenopausal vs. older women) and exercise interventions (i.e. skeletal loading characteristics). Most of the literature, usually based in animal models, published thus far supports the notion that greater strain magnitudes and unusual strain distributions provide the most effective stimuli for bone formation [6]. However, older adults are unable to sustain high impact-loading regimens without elevated risk of injury, and compliance with high-impact loading regimens is extremely difficult. Thus, designing the appropriate exercise prescription is challenging, along with the limited information provided by the current guidelines [7, 8] exclusively based on data from studies examining the isolated effect of different exercise protocols in adults aged 60 years and older. Moreover, only a few studies have addressed the skeletal response to long-term exercise training in older men [9-13], thus, scarce information is available for the purpose of prescribing optimal exercise regimes for older men. Indeed, even with no change in current incidence rates, it has been estimated that the number of hip fractures will double to 2.6 million by the year 2025, with a greater percentage increase in men than in women [14].

In addition, experimental animal studies have recently implicated inflammation in the pathogenesis of osteoporosis [15], mediated by inflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin (IL)-6. The effect is primarily driven on the differentiation and activity of the bone-resorbing cell, the osteoclast [16]. On the other hand, interferons (IFN) seem to suppress osteoclast differentiation and inhibit bone loss [16]. Receptor activator of nuclear factor kappa B ligand (RANKL) is the key osteoclastogenic cytokine as osteoclast formation requires its presence or its priming of precursor cells. Osteoprotegerin (OPG) is a decoy receptor for RANKL and can block RANKL/RANK interactions [17]. It is established that pro-inflammatory cytokines suppress OPG expression while simultaneously enhancing RANKL expression [16]. In addition, evidence exists to support a relationship between regular exercise and improvements in systemic low-grade inflammation [18, 19], even in old age [20], along with *in vitro* and *in vivo* experiments suggesting that mechanical stimulation can inhibit osteoclast formation and activity by increasing OPG/RANKL ratio [21, 22]. Nevertheless, to date there are no reports documenting whether changes in bone-related inflammatory cytokines are associated with alterations in BMD in older adults after prolonged exercise training.

In addition, basic and clinical studies have established the relevance of biochemical markers of bone metabolism, which show an early response following treatment compared with BMD, for

monitoring therapeutic response and efficacy on individual patients [23]. A combination of markers have been used to evaluate the rate of bone remodeling, including measuring predominantly osteoblastic or osteoclastic enzyme activities or assaying bone matrix components in blood and/or urine [23]. However, only few studies focused on older adults have addressed the influence of long-term exercise on those biomarkers [24, 25].

Therefore, the aim of the present study was to analyse the alterations in balance as a key factor associated with fall risk, BMD, bone-related inflammatory cytokines, and different biomarkers of bone metabolism, in older men and women after a 32-week exercise training programme. The results obtained would contribute to a better understanding of how combined impact loading intervention can interact with the physiological systems associated with bone remodelling in older men *vs.* women.

2. Materials and Methods

2.1 Subjects and experimental design

Subjects were recruited through advertisements in Porto area newspapers for participation in this university-based study. A total of 55 Caucasian older adults volunteered to participate in the study. The eligible subject pool was restricted to older adults with the following characteristics: aged 60-85 years, community-dwelling status, not engaged in regular exercise training in the last year, lack of use of bone-acting drugs and nutritional supplements known to affect bone metabolism (such as vitamin D and calcium) within the previous year, and lack of and significant sensory/cognitive impairment or medical conditions that contraindicated participation in exercise. On the initial screening visit, all participants received a complete explanation of the purpose, risks, and procedures of the investigation and, after signing a written consent form, the past medical history and current medications of the subjects were determined. Participants were instructed to continue their daily routines and to refrain from changing their physical activity (PA) levels during the course of the experiment.

The baseline characteristics of the participants are given in Table 1. The study was carried out in full compliance with the Helsinki Declaration, and all methods and procedures were approved by the institutional review board.

2.2 Measurements

Participants were tested on two occasions: the first assessment was conducted prior to the beginning of training (last week of September 2009) and the second evaluation took place after eight months of training (first week of June 2010).

2.2.1 Bone and body composition

BMD was measured using dual-energy X-ray absorptiometry (DXA) (QDR 4500A, Hologic, Bedford, MA) at the lumbar spine (L1–L4) and proximal femur on the nondominant side using standard protocols. To minimise interobserver variation, the same investigator carried out all analyses. Bone phantoms were scanned daily, and coefficients of variation (CV) were verified before and during the experimental period to ensure assessment reliability.

Total body scans were taken using the same DXA instrument. All scans were performed by the same technician using standard procedures, as described in the Hologic user's manual. Scans were analysed for total lean mass and percent body fat mass. Precision for DXA BMD measures of interest are 0.8–1.1% in our laboratory with CV (standard deviation/mean) calculated from duplicate scans of older adults. CV-values for lean mass and percent body fat, mass are 1.1% and 3.1%, respectively.

Height and body mass were recorded using a portable stadiometer and balance weighing scales, respectively. Body mass index (BMI) was calculated using the standard formula: mass (kg)/height^2 (m).

2.2.3 Balance performance measure

Mobility/dynamic balance was assessed using the 8-foot Up and Go Test (8-ft UG test) [26]. Before starting the test, participants remained seated and rested for five minutes. The score corresponded to the shortest time to rise from a seated position, walk 2.44 m (8 feet), turn, and return to the seated position, measured to the nearest 1/10th s. Two attempts were allowed, with one minute of rest between, and the best performance was used for analysis.

2.2.4 Blood sampling and analysis

Fasting venous blood samples were drawn between 8 a.m. and 10 a.m. always on Mondays to ensure at least 2 days without exercise training. After collection, blood samples were collected in tubes containing EDTA, and serum samples were clotted at room temperature for 90 min and were then centrifuged for 10 minutes at 1000 x g. Samples were aliquoted and stored at -80 °C until analysis.

Serum N-MID Osteocalcin (OC) was measured using an electrochemiluminescence immunoassay (ECLIA) on a Cobas E module (Roche Diagnostics, Penzberg, Germany), and data were analyzed on a Elecsys 2010 platform. For serum C-terminal telopeptide of Type I collagen (CTX) the serum CrossLaps ELISA kit (Immunodiagnostic Systems Ltd, Boldon, UK) was used. Serum concentrations of IL-6, TNF-α, and IFN-γ (Human 3-plex Cytokine panel), serum OPG (Human bone panel 1A), and serum RANKL (Human RANKL Single Plex) were measured using MILLIPLEX™ MAP kits (Millipore, St. Charles, MO) for Luminex 200 technology (Luminex Corporation, Austin, TX). Raw data (mean fluorescence intensity, MFI) were analyzed using IS™ 2.3 software (Luminex Corporation, Austin, TX). All analyses were performed according to the manufacturers' protocols. All standards and samples were measured in duplicate. All these assay methods have CV-values (intra- and inter-assay) <9%.

Capillary blood samples were collected on the same occasion from the earlobe using a 50-µl lithium heparin-coated capillary tube and immediately assayed using the Cholestech LDX® Analyzer (Cholestch Corporation – Hayward, CA, USA) for determination of hs-CRP.

2.2.5 Lifestyle behaviours and clinical status

A baseline self-administered questionnaire to assess the impact of present and past lifestyle choices was completed by interview to avoid misinterpretation of items and/or skipping of questions. The questionnaire included information regarding education; marital status; fall and fracture history; medical history; current medical conditions; medication use; current and past PA; age at menarche; menopause status; and current and past smoking.

2.2.6 Daily PA

The Actigraph GT1M accelerometer (Manufacturing Technology, Fort Walton Beach, FL) was used as an objective measure of daily PA, as described in our previous study [3]. For both test periods (pre- and post-training), four files had only two valid days. Those 4 participants were contacted and agreed to wear the accelerometer again for seven days (one week later than the rest of the group). The average daily moderate to vigorous PA (MVPA), number of steps, and daily activity counts per minute (cpm) were analysed.

2.2.7 Nutritional assessment

Nutritional status was assessed using 4-day diet records over three weekdays and one weekend day. To ensure standardisation of the dietary records, a dietician gave individual instruction to the subjects concerning how to fill out the diet records and assess food serving sizes. Diet records were analysed using Food Processor Plus® (ESHA Research, Salem, OR), which uses the table of food components from the U.S. Department of Agriculture. Some traditional Portuguese dishes were added based on the table of Portuguese food composition. Total caloric, protein, calcium, phosphorous, vitamin D, and caffeine intakes were compared between men and women.

2.3 Exercise protocol

The 32-week combined loading training involved odd-impact loading training performed one day per week (Wednesdays) and high-magnitude joint reaction force loading through resistance training performed two days per week (Mondays and Fridays). Each session lasted approximately 60 min, and all sessions were accompanied by appropriate music relevant to the required activity and participants' age. All sessions were led by three physical education instructors specialized in PA for older adults, and supervised by the researchers; sessions were conducted at University of Porto - Faculty of Sport facilities.

The odd-impact training was designed to load bones with intermittent and multidirectional compressive forces, introducing atypical and novel stress on the bone, and to improve

neuromuscular function. Each training session included: a 10-min light stretching and warm-up exercise; 15 min of weight-bearing activities, consisting of stepping exercise at a speed of 120–125 beats per minute using a 15-cm-high bench, bounding exercises, and heel-drops performed on a hard surface. A heel-drop consists of raising the body weight onto the toes and then letting it drop to the floor, keeping the knees locked and hips extended; muscular endurance exercises performed concentrically and eccentrically for about 10 min, involving squats while wearing weight vests, hip flexors, extensors, and abductors; knee flexors and extensors and upper body exercises performed using elastic bands and dumbbells; 10 min of balance training with static and dynamic exercises (e.g., walking in a straight line, walking heel to toe, using additional resources such as ropes, sticks, balls, and balloons), 10 min games where movements included directional elements that the body is not normally accustomed to, and agility training aimed at challenge hand–eye coordination, foot–eye coordination, dynamic balance, standing and leaning balance; and 5 min of stretching. For weight-bearing and strength exercises, the repetitions were increased from eight to 15 and the number of sets increased to three.

Resistance exercise training sessions involved the following: a standardised warm-up period (8–10 min) on a bicycle ergometer (Bike-Max; Tectrix, Irvine, CA) and/or rowing ergometer (Concept II, Morrisville, VR) at low intensity and some stretching exercises; specific resistance training period (30–40 min) that included leg press and leg extension, seated leg curl, hip abduction, double chest press, lateral raise, overhead press, and abdominal machine; and a cool-down period (5–10 min) that included walking and stretching exercises. Subjects exercised on variable resistance machines (Nautilus Sports/Medical Industries, Independence, VA). To minimise fatigue, the exercises for the upper/lower parts of the body were performed in a non-consecutive way, with a rest period of approximately two minutes between each set. Each repetition lasted three to six seconds, involving a period of at least two minutes between the two sets of 10–12 repetitions at 60-70% of 1RM. Training intensity was gradually increased during the first four weeks. Participants underwent a 2-week familiarisation period with the equipment and the exercises. The intensity of the training stimulus was initially set at 60% of one-repetition maximum (1RM), as determined at week 2, with a work range of three sets of 12 to 15 repetitions. Subjects then progressed from 75% to 80% of 1RM at a work range of six to eight repetitions (three sets) and remained at this level until the end of the programme. Training was continuously monitored by heart rate monitors (Polar Vantage XL, Polar Electro Inc., Port Washington, NY) and ratings of perceived exertion (Borg's 10-point psychometric scale) [27]. 1RM tests were performed every two weeks for the first month and then every four weeks until the end of the programme. Between these tests, the load was increased for those subjects who were able to easily complete 12 or more repetitions for both sets.

The three research assistants were responsible for warm-up, cool down, and stretching exercises; the monitoring of correct lifting form; the appropriate amount of exercise and rest intervals; the maintenance of daily exercise logs; and the progression of the exercises. Subjects were also encouraged to exercise with a training partner to provide additional motivation.

Exercise compliance was defined as the number of exercise sessions reported divided by the number of maximum exercise sessions possible.

2.4 Statistical analysis

All statistical analyses were performed using PASW Statistics (version 18; SPSS, Inc., Chicago, IL) for Windows with a significance level of 0.05. Data were checked for distribution, and the means ± SD were calculated. Primary outcomes were changes from baseline in response to both 8-month interventions in balance, BMD and serum bone metabolism markers and inflammatory cytokines. Secondary outcomes included 8-month changes from baseline in dietary intake, daily PA, and body composition (BMI, percent fat mass, and lean mass). The results were analysed on an intention-to-treat basis, and missing data due to lack of follow-up (the method assumed data were missing at random) were replaced using the process of multiple imputation. This method has been adapted to the analysis of longitudinal data [28]. Between-group comparisons of continuous variables were performed using t-tests. Pearson's correlations coefficients were used to calculate relationships among BMD of the femoral neck, total hip, and lumbar spine, body composition, bone biomarkers, and inflammatory markers. Correlation analyses were also used to determine relationships among the change (8 months – baseline) of the above variables.

The delta percentage was calculated with the standard formula: % change = [(posttraining score − baseline score)/baseline score] ×100, and the effect size (ES) for within-subjects ($M_1 – M_2/S_1$), where M_1= pre-test mean, M_2= post-test mean and S_1=pre-test standard deviation was also calculated. An ES of 0.2 or less is considered small, an ES around 0.5 is moderate, and an ES of 0.8 or greater is large [29].

A two-way (group and time) factorial ANOVA, with repeated measures on one factor (time), was performed for differences in main effects and time by group interactions for each dependent variable. Main effects were considered when interactions were not significant. When significant interactions were found, Bonferroni post hoc tests were used to determine significant differences amongst mean values.

A power analysis based on a formulation of 75% power, an effect size of 0.5 for overall muscle strength, balance and BMD from previous studies, and a significance level of 0.05 for a one-tailed test deemed that a sample of 23 per group was sufficient to address the research questions.

3. Results

Subjects

Eight subjects did not meet selection criteria due to use of medication known to affect bone metabolism (n=3), use of hormone replacement therapy (n=2), and current involvement in

water-based activities (n=3). Forty of the original 47 subjects (women=24, men=23) who underwent the initial assessment completed the study (women=20, men=20). Dropout rates were similar amongst men and women. Three participants dropped out because of surgery, two participants dropped out due to medical issues unrelated to the intervention, and two other subjects because of personal reasons. One-hundred percent compliance to the exercise sessions was set at 96 training sessions. Excluding dropouts, mean compliance to exercise sessions was 82.6% (60.0–100%). There were no exercise- or assessment-related (pre- and post-training) adverse events. In comparison to individuals who completed the trial, those who failed to provide follow-up data had lower daily MVPA level and lower performance in balance test. No significant differences in the remaining baseline measurements were found, including age, body weight, inflammatory or bone-related variables. Demographics and descriptive parameters of all groups are listed in Table 1. Of the participants, 45% of the participants were overweight, almost half of them had hypertension, women never smoked, and only a small proportion of men (6%) had a history of cigarette smoking. On average, participants obtained 78 minutes of MVPA per day. Compared with men, women had significantly lower weight (p<.001), lower caffeine intake (p=0.025), and significantly less BMD and T-score values.

Table 1 Baseline characteristics of the sample

Variable	Women (n = 24)	Men (n = 23)	p value
Age (years)	68.2 ± 5.7	68.2 ± 5.2	0.876
Education (years)	7.9 ± 4.5	8.4 ± 3.6	0.373
Weight (kg)	64.2 ± 10.2	83.0 ± 11.7	<0.001
BMI (kg/m^2)	27.7 ± 4.2	29.7 ± 3.5	0.134
Number of routine medications	1.9 ± 1.8	2.2 ± 1.6	0.459
Diet			
Energy intake (kcal/day)	1444.6 ± 345.4	1618.3 ± 496.5	0.169
Protein intake (g/day)	68.7 ± 14.6	71.5 ± 19.4	0.578
Calcium intake (mg/d)	643.7 ± 337.9	658.3 ± 253.3	0.868
Phosphorus intake (mg/day)	988.7 ± 299.6	1049.1 ± 319.2	0.507
Vitamin D intake (µg/day)	1.7 ± 1.9	1.5 ± 1.2	0.619
Coffee intake (mL/day)	62.4 ± 57.0	98.2 ± 48.5	0.025
Daily Physical Activity			
MVPA (min/day)	80.4 ± 30.6	91.9 ± 31.5	0.294
Daily counts per minute	377.6 ± 123.6	418.1 ± 139.1	0.383
Daily step count	9255.7 ± 3195.2	10629.1 ± 9668.4	0.614
Bone mineral density			
Lumbar spine (g/cm^2)	0.848 ± 0.121	1.039 ± 0.173	<0.001
Femoral neck (g/cm^2)	0.687 ± 0.108	0.817 ± 0.099	0.001
Lumbar spine (T-score)	-1.8 ± 1.2	-0.4 ± 1.6	0.011
Femoral neck (T-score)	-1.5 ± 1.0	-0.7 ± 0.9	0.014

BMI body mass index, *MVPA* moderate to vigorous physical activity

Secondary outcomes

Total energy intake was similar amongst men and women at baseline and during the period of intervention. Energy intake was 1,530 ± 430 kcal/day at baseline and 1,452 ± 463 at eight

months. Dietary protein, phosphorus, caffeine, calcium, and vitamin D intake measured with a 4-day dietary record, remained unchanged after 8 months of intervention (data not shown).

In total, pre- and post-training data from all participants were included in the analysis (47 files with seven valid days, 7 files with 6 valid days, and 11 files with 5 valid days). No significant changes in MVPA level were observed at eight months. There was no significant interactive (p=0.210) or main effect of group (p=0.102) and time (p=0.326) on changes in PA. Changes in MVPA were not related to changes in the primary outcomes.

No significant interaction occurred for body composition in response to exercise intervention (Table 2). There was a main effect of group on lean mass (p< 0.001) and percent fat (p< 0.001). Thus, women had significant lower lean mass and greater percent fat on both time-points. However, the ES for all body composition variables were low (ES<0.2) and changes in body composition were non-significant. Changes in body composition were not associated to changes in the primary outcomes.

Changes in balance and BMD

There were no significant interactions between group and time on all measurements of balance and BMD (Table 2 and Table 3). A significant main effect of time for all balance and bone variables was observed, excepting for femoral neck BMD. Accordingly, both men and women improved the time to perform the balance test and improved BMD at several bone sites, including lumbar spine, total hip, trochanter, and intertrochanteric region. However, the magnitude of the effect observed on balance was moderate in women, and low in men. In addition, ES for BMD sites were low (ES<0.2). A significant main effect of group was also observed for all variables; thus, as expected, women had lower BMD at both time-points, and significant lower performance on the up and go test at baseline and after training.

Table 2 Pre- and post-training values, and effect sizes (ES) for body composition and dynamic balance.

	Women			Men					
Variable	Pretraining	Posttraining	ES	Pretraining	Posttraining	ES	p (Group)	p (Time)	p (Int)
BMI (kg/m^2)	28.62±4.13	28.47±4.35	0.04	29.33±3.4	29.0±3.42	0.09	0.572	0.057	0.538
LM (kg)[a]	38.38±4.83	38.45±5.05	0.01	54.87±5.86	55.30±6.24	0.05	<0.001	0.111	0.258
FM (%)[a]	37.83±5.82	37.63±5.85	0.04	27.59±5.21	27.17±5.08	0.08	<0.001	0.090	0.552
8ft UG (s)[a]	5.33±1.04	4.74±0.54	0.56	4.55±0.74	4.35±0.51	0.28	0.004	<0.001	0.059

[a] Significant difference between groups at baseline and post-training, p < 0.05.

BMI body mass index, *8 ft UG* 8-foot Up and Go Test, LM Lean mass, FM Fat mass, Int interaction

Changes in inflammatory cytokines

Inflammatory markers were not significantly different between groups at baseline. There was a significant treatment effect (decrease) for IL-6, IFN-γ, and hs-CRP (Table 3). A significant group

x time interaction only for IL-6, thus a different response between males and females was evident over time, supported by the significant decrease observed in the male group (ES=0.52 *vs.* ES=0.09). There was no significant interaction or main effects of group and time in TNF-α.

Changes in bone biomarkers

The 8-month exercise training did not significantly change in both bone turnover markers (OC and CTX) and in OPG, RANKL and their ratios (Table 3). OPG was significantly greater ($p<0.05$) in the female group compared to the male group at baseline and post-training.

Correlations

At baseline, lean mass was positively correlated with femoral neck, total hip and lumbar spine BMD. OPG was negatively correlated with femoral neck and total hip BMD. There were no significant correlations between inflammatory markers and baseline BMD at any bone site. The change (8 months–baseline) in TNF-α was negatively correlated with the change in lumbar spine BMD ($r=-0.30$, $p=0.047$). The change in OC correlated significantly with the change in CTX ($r=0.50$, $p<0.001$), and with hs-CRP ($r=0.33$, $p=0.022$). The change in CTX also correlated significantly with IL-6. The change in bone metabolism markers did not correlate significantly with change in BMD at any site.

Table 3 Eight-month changes and ES for proximal femur and lumbar spine BMD, serum bone-related and pro-inflammatory markers

	Women			Men			p	p	p
Variable	Pretraining	Posttraining	ES	Pretraining	Posttraining	ES	(Group)	(Time)	(Int)
FN (g/cm^2)[a]	0.715±0.119	0.705±0.104	0.08	0.822±0.113	0.821±0.115	0.01	0.002	0.241	0.406
Troch (g/cm^2)[a]	0.640±0.081	0.648±0.080	0.10	0.773±0.112	0.774±0.114	0.01	<0.001	0.032	0.070
Inter (g/cm^2)[a]	1.031±0.142	1.041±0.139	0.07	1.169±0.165	1.172±0.163	0.02	0.005	0.038	0.276
T hip (g/cm^2)[a]	0.864±0.108	0.872±0.111	0.08	1.004±0.140	1.006±0.138	0.01	0.001	0.021	0.129
LS (g/cm^2)[a]	0.877±0.122	0.896±0.129	0.15	1.051±0.161	1.065±0.172	0.08	<0.001	<0.001	0.427
OC (ng/mL)	14.82±3.64	15.43±4.12	-0.17	14.08±2.87	13.75±2.80	0.12	0.195	0.717	0.225
CTX (ng/mL)	0.38± 0.14	0.38±0.15	0.01	0.37±0.12	0.36±0.12	0.12	0.722	0.571	0.667
OC/CTX	42.91±15.35	43.83±12.55	-0.06	39.45±7.92	40.67±10.88	-0.15	0.309	0.450	0.915
OPG (pg/mL)[a]	514.61±117.57	503.45±118.85	0.09	432.80±126.10	423.81±107.64	0.07	0.018	0.294	0.909
RANKL (pg/mL)	29.64±13.82	26.53±14.64	0.23	27.20±9.72	26.53±9.89	0.07	0.722	0.090	0.269
OPG/RANKL	30.38±39.60	31.49±36.85	-0.03	17.64±6.83	19.66±12.32	-0.30	0.122	0.557	0.865
IL-6 (pg/mL)	1.18±0.81	1.11±0.91	0.09	1.62±1.25	0.97±0.84[b]	0.52	0.538	0.042	0.013
TNF-α (pg/mL)	7.30±2.46	7.28±2.16	0.01	7.39±2.05	7.99±2.52	-0.30	0.538	0.164	0.149
IFN-γ (pg/mL)	0.75±0.51	0.48±0.36	0.54	0.63±0.43	0.44±0.21	0.45	0.393	0.002	0.393
Hs-CRP (mg/L)	3.06±2.27	2.54±1.81	0.23	2.53±1.95	1.63±1.01	0.46	0.133	0.006	0.428

[a] Significant difference between groups at baseline and post-training, $p < 0.05$.
[b] Significantly different from baseline, $p < 0.05$.
BMD bone mineral density, *CTX* C-terminal telopeptide of Type I collagen,*OC* osteocalcina, FN femoral neck, *Hs-CRP* high sensitive-C reactive protein, *Int* interaction, *IFN* interferon, *IL* interleukine, *Inter* intertrochanteric region, LS lumbar spine, *OPG* osteoprotegerin, *RANKL* receptor activator of nuclear factor kappa B ligand, *TNF* tumor necrosis factor, *T* total, *Troch* trochanter.

4. Discussion

Regular exercise training is consistently linked with a varied range of health-related benefits, including improvements in low-grade inflammation, BMD and bone metabolism. In fact, increased inflammation seems to be associated with a reduction in BMD, as key cytokines are associated with critical factors for bone remodelling [16]. However, few comprehensive efforts have been made to characterise the relationship between changes in BMD, bone metabolism, and inflammatory response after long-term exercise training in older adults. We found that the 32-week resistance exercise combined with weight-bearing exercise training improved balance (fall risk factor), BMD and favourably modulate inflammatory markers in older adults. No changes were observed in OPG, RANKL, and OC and CTX (bone formation and resorption markers, respectively) after exercise.

Previous studies report that exercise training is associated with balance improvements in healthy older adults [2, 3, 30, 31], reinforcing the notion that exercise training has the potential to reduce fall risk in elderly people [32]. In the present study, both older men and women significantly increased dynamic balance, which is in line with the prevailing evidence that exercise protocols that include a specific strengthening and balance component are the most effective exercise interventions for balance improvements compared with other modes of training [33].

Decreased BMD is common in older age, and it is assumed that it is primarily the reduced osteoblastic activity and number (inability of bone narrow stromal cells to deposit adequate amounts of bone to compensate for the amount removed by osteoclasts) that lead to decreased bone mass in the aging skeleton [34]. In addition to BMD measures, biochemical markers provide dynamic information on bone turnover that is not provided by static bone mass measurements [35]. Our results showed that resistance exercise plus weight-bearing multicomponent exercise significantly increased total hip, trochanter, intertrochanter and lumbar spine BMD in both men and women, whereas no significant effects were observed on bone turnover markers (OC and CTX), and serum levels of OPG and RANKL. Evidence regarding exercise effects on bone mass and metabolism has been mostly based on women, and few studies have focus on older adults (age>60y), particularly men [36]. Human studies examining the effects of long-term exercise interventions in older men have shown positive results on both femoral neck and lumbar spine BMD [9, 10, 12, 13, 37]. All previous studies were based on evidence from resistance exercise, and training lasted between 6 to 18 months, which is consistent with our intervention. Our data also revealed that the relative improvements in BMD with training were similar for both men and women, which supports the findings of Bemben et al. [37]. We examined the effect of a combined training protocol, including odd-impact exercise 1 day/week (such as aerobic or step classes, bounding exercises, agility exercises and games, and impact activities) with resistance exercises 2 days/week, and utilized site-specific exercises to apply mechanical loads to the skeleton. The positive results mediated by this type of exercise

intervention are in agreement with results from our recent meta-analysis [36]. We reported that odd-impact protocols were effective in increasing BMD at lumbar spine and femoral neck in older adults, and combined loading studies of impact activity mixed with resistance training were effective at lumbar spine and no inconsistency existed among these trials [36]. However, results were based on a disproportionate emphasis toward women. Thus, evidence to support the efficacy of exercise training on bone health in older men is needed.

Data from exercise training and bone turnover markers are however inconsistent, with some studies showing positive effects of exercise in attenuating bone turnover in aging adults [13, 38, 39] while others showed no effects on both bone formation and resorption markers [40, 41]. Our previous work also demonstrated no changes in serum OPG and RANKL levels and their ratio, after 8 months of exercise training [2]. Similarly, Esen et al. [42] reported no significant changes in OPG levels after a 10-week walking programme in middle-aged men, and a study using human cell lines showed that mechanical stimulation had no affect RANKL [21]. Our findings did not support the hypotheses that mechanical load may induce down-regulation of RANKL [22, 42-44]. Findings of the present study regarding changes in both bone turnover markers did not reach level of statistical significance, which paralleled with the data reported by Ryan et al. [45] and Bemben et al. [25]. However, the ratio of OC to CTX increased 5% and the ratio of OPG to RANKL increased 15%, which may suggest a positive bone metabolism change. The lack of exercise effect on bone metabolism may be attributed to the limited number of serum measurements (only at baseline and after 32 weeks). Previous exercise-based studies on bone metabolism were mostly short-term, lasting commonly 16 weeks, as bone marker responses to training are more rapid than BMD responses [46]. Karabulut et al. [38] found a significant increase in bone specific alkaline phosphatase (B-ALP) and B-ALP to CTX ratio after 6 weeks of resistance exercise in older men.

Although the anti-inflammatory effect of exercise training is assumed to be mediated by decreased inflammatory cytokines such as IL-6, TNF-α, IFN-γ, and CRP [19, 47], the association between inflammation and bone metabolism after long-term exercise is less clear.

Previous cross-sectional studies have linked high CRP levels with lower BMD [48], higher levels of bone turnover markers [49], and greater risk of fracture [50]. Others have also associated elevated levels of pro-inflammatory cytokines with increased risk of bone loss [16, 51]. The circulating levels of IFN-γ, and hs-CRP decreased in both men and women, and IL-6 significantly decreased only in men in response to the present exercise training program (Table 3). Of note, the observed decrease in inflammation was independent of weight loss, as no significant reductions on body weight or fat mass occurred after the 32-week exercise intervention. Indeed, most evidence concerning the exercise effect on inflammation had consistently focused on obese and/or type 2 diabetic subjects [52-55]. Nevertheless, and in accordance with our results, some previous studies involving elderly subjects found that exercise lead to a significant decrease in IL-6 [56, 57] and CRP [20, 58, 59], and no significant changes on TNF-α [20, 56, 60]. The mechanisms by which pro-inflammatory cytokine mediate

bone damage have been postulated [16]. In brief, TNF exerts its effect on osteoclastogenesis by acting directly on osteoclast precursors, as well as indirectly, by upregulating the production of macrophage colony-stimulating factor and RANKL on mesenchymal cells [61, 62]. IL-6 can upregulate RANKL and thus indirectly support osteoclast formation via the interaction with mesenchymal cells [63]. However, our results indicate that the changes in pro-inflammatory cytokines were not associated with changes in serum RANKL levels. The only significant association (negative) was found between the change in TNF-α and change in lumbar spine BMD (r= -0.30, p= 0.047), which may partially corroborate the negative effect of TNF on bone formation. The suppressive function of IFN-γ on osteoclast differentiation is also based on interference with RANKL signaling [64]. It is suggested that a low level of IFN-γ strongly inhibits osteoclast formation, even in the presence of an excess amount of RANKL [64]. However, exercise-associated changes in IFN-γ have been poorly studied. The results from prospective training studies are controversial, as the IFN-γ production have been reduced [65] or did not change [55] following a 8-week exercise training program in young individuals. Therefore, we interpret the reduction of IFN-γ in our study as an indication that exercise training reduces inflammation that may mediate a positive effect on osteoclast formation. However, no association were found between baseline and change in IFN-γ and bone biomarkers or BMD.

Some limitations of this study should be acknowledged. While we did not conduct a randomized controlled trial (RCT), the improvement in femoral neck (0.6%) and lumbar spine BMD (1.7%) demonstrated in this study is consistent with RCTs [2, 3, 37]. It should be noted that controlled trials may overestimate treatment effects compared to RCTs [66], which is not perceptible in the present study. Moreover, the modest magnitude of BMD improvements (albeit significant) in response to training may had caused changes in bone geometry [67], not measured by DXA, which translate to gains in bone strength. However, DXA is the method most commonly used to measure BMD area (g/cm^2) because of its speed, precision, low radiation exposure and availability of reference data [68].

Our study prvides novel data regarding the relationship between exercise training and bone changes, including BMD measurements and bone metabolism markers, and inflammatory cytokines in this particular population. Another unique aspect of our study was the inclusion of both men and women which allowed gender comparisons of the responses to training. Moreover, this study has considered the possible influence of critical confounding variables such as daily PA levels objectively measured by accelerometers as well as dietary intake.

In conclusion, the results further support the beneficial role of long-term exercise training on bone mass and low-grade inflammation. We have also demonstrated that exercise training elicits significant gains in balance. The relationship between exercise and changes in BMD as well as bone metabolism markers, and inflammatory response needs to be further explored, given the public health importance of bone fragility and susceptibility to falls with aging.

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Paper V

AGE
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Exercise effects on bone mineral density in older adults: a meta-analysis of randomized controlled trials

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Abstract The purpose of the study was to assess the effects of exercise interventions with different impact loading characteristics on lumbar spine (LS) and femoral neck (FN) bone mineral density (BMD) in older adults. We searched electronic databases and hand searched selected journals up to February 2011 for randomized controlled trials (RCTs) investigating the effects of impact exercise interventions on LS and FN BMD in older adults. Exercise protocols were categorized according to impact loading characteristics. Weighted mean difference (WMD) meta-analyses were undertaken. Heterogeneity amongst trials and publication bias was tested. Random-effects models were applied. Trial quality assessment was also undertaken. Nineteen RCTs, including 1577 subjects, met the inclusion criteria. Twenty-two study group comparisons reported BMD data at the LS. Meta-analysis showed a significant change in BMD at this site (WMD 0.011 g/cm^2, 95% CI 0.003 to 0.020; p=0.007), although results were moderately inconsistent (I^2= 52.2%). BMD data at the FN were available from 19 study group comparisons among older adults. Results were inconsistent (I^2=63.6%) in showing a significant positive effect of exercise on BMD at this site (WMD 0.016 g/cm^2, 95% CI 0.005 to 0.027; p=0.004). Combined loading studies of impact activity mixed with high-magnitude joint reaction force loading through resistance training were effective at LS (WMD 0.016 g/cm^2, 95% CI 0.002 to 0.036; p=0.028), and no inconsistency existed among these trials. Odd-impact protocols were also effective in increasing BMD at LS (WMD 0.039 g/cm^2, 95% CI 0.002 to 0.075; p=0.038) and FN (WMD 0.036 g/cm^2, 95% CI 0.012 to 0.061; p=0.004), although heterogeneity was evident (I^2= 87.5% and I^2=83.5%, respectively). We found consistency among results for low-impact and resistance exercise studies on LS and FN, although non-significant BMD changes were evident amongst these types of protocols at any site and amongst the RCTs that provided a combined loading impact exercise at FN. Funnel plots showed no evidence of publication bias. Trial quality was moderate to high. The findings from our meta-analysis of RCTs support the efficacy of exercise for increasing LS and FN BMD in older adults.

Keywords Systematic review · Meta-analysis · Bone density · Exercise · Aging

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Introduction

Aging is linked to a decreased osteoblast activity, increased osteoclast activity and diminished differentiation potential of bone marrow stem cells due to a relative decline in trophic factors (e.g. oestrogen, IGF-1, vitamin D) favoring local expression of molecules such as interleukins and TNF-α (Khosla and Riggs 2005). As

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a result, the amount of bone tissue is reduced, which consequently motivates bones to become weaker, commonly leading to osteoporosis. This is a common, serious, and disabling condition due to the inherent association with low-energy trauma or fragility fractures. Hip and vertebral fractures have serious complications such as chronic pain, disability, diminished quality of life and premature death (Dhanwal et al. 2011; Johnell and Kanis 2005) and therefore have become a major and growing problem as elderly population is increasing in every geographical region (Dhanwal et al. 2011; Cooper et al. 2011). Like women, older men become susceptible to age-related bone mineral loss, which continues for the remainder of life, and vertebral and hip fracture rates also increase with advancing age.

The number of hip fractures that occur each year in the world has been estimated by Gullberg et al. (1997) to be 1.25 million (338,000 in men and 917,000 in women) in 1990 and is predicted to rise by 310% in men and 240% in women by 2025. Additionally, there are severe economic consequences of fragility fractures, as the combined annual cost has been estimated to be $20 billion in the USA and €30 billion in the European Union (Cummings and Melton 2002). Thus it is worthwhile to prevent major osteoporotic fractures, namely hip and vertebral fractures, with intervention.

Physical exercise has been advised as a preventive and therapeutic strategy against aging-induced bone weakness (Schwab and Scalapino 2011), although it has been also described that osteogenic responsiveness to mechanical loading declines with age (Lanyon and Skerry 2001). The effects of exercise on bone mass appear to be attributed to the activation of osteocytes, which in turn alter the balance between bone resorption and formation, favoring modeling, if mechanical loading creates strains of sufficient magnitude (Hsieh et al. 2001). The current American College of Sports Medicine (ACSM) position stand on exercise and physical activity for older adults (Chodzko-Zajko et al. 2009) summarizes published research with respect to the known benefits of exercise on bone health mostly based on postmenopausal women (mean age are commonly set between 55 and 65 years), although different results may be expected in elderly subjects. Recommendations include aerobic exercise training such as walking (low-intensity weight bearing activity) and stair climbing/descending, brisk walking, walking with weighted vests or jogging (higher-intensity bone loading activities) which may be effective in counteracting age-related declines in bone mineral density (BMD) in postmenopausal women, and high-intensity resistance exercise training as it seems to preserve or improve BMD relative to sedentary controls (Chodzko-Zajko et al. 2009; Nelson et al. 2007). During the past years, meta-analyses of exercise effects on bone mass have focused on premenopausal and postmenopausal women (Martyn-St James and Carroll 2009; Berard et al. 1997; Martyn-St James and Carroll 2008; Kelley et al. 2001). Results confirmed that exercise may have a positive influence on the skeleton, by increasing or maintaining BMD at the loading sites, although the type of exercise, the skeletal loading characteristics of the different impact exercise interventions and the bone site measured lead to contrasting results (Martyn-St James and Carroll 2009; Berard et al. 1997; Martyn-St James and Carroll 2008; Kelley et al. 2001). Despite the importance of bone density in the elderly, according to our knowledge no previous systematic literature reviews and meta-analyses of the efficacy of physical exercises exclusively on older adults have been performed. Thus, we conducted a systematic review and meta-analysis of randomized controlled trials (RCTs) to assess the effects of exercise on lumbar spine and femoral neck BMD in older adults.

Methods

We carried out our systematic review using a pre-specified protocol, devised according to the guidelines of the Cochrane Collaboration (Higgins and Green 2009).

Criteria for considering studies for this review

The inclusion criteria for this study were (1) randomized controlled trials (RCTs), (2) exercise as the only intervention, (3) older adults aged ≥60 years or whose mean age is ≥65 years, (4) data for one or more of the following variables provided: lumbar spine BMD and femoral neck BMD, (5) studies published in English language journals, (6) comparative control group and (7) exercise intervention lasting a minimum of 16 weeks.

Only information that met the above criteria was included in our analysis. We did not include abstracts and conference papers from national meetings because of the paucity of data provided as well as the inability to obtain complete data from the authors. Studies published in

non-English-language journals were also not included due to potential errors in the translation and interpretation of findings. Intervention exercise or physical activity trials were defined as weight-bearing exercise (meaning a structured, force-generating activity which loads the skeletal regions above the stimuli provided by activities of daily living (MacKelvie et al. 2002; Hind and Burrows 2007)). Weight-bearing exercises can include aerobics, resistance, endurance training, circuit training, jogging, jumping and other modalities that generate impact to the skeleton. Habitual recreational activity without any specific intervention or supervised activity known not to affect bone (sham exercise) was accepted as activity for the control participants.

We excluded uncontrolled trials, cross-sectional and case-control studies and animal investigations. Among RCTs, we excluded studies in which exercise was combined with other interventions or treatments, such as anti-osteoporotic medication and nutritional or hormonal therapies, as both effects could not be separated. We also excluded studies that included participants (even some) already taking/engaged in those treatments/interventions. Interventions involving multiple behaviors, such as diet plus exercise, were not included although the use of supplements of calcium and vitamin D was acceptable, unless equally distributed between study arms in a given trial. Studies of exercise interventions for individuals institutionalized and/or with specific diseases or conditions or with severe physical disabilities or presence of severe frailty were not included. Extremely low-impact exercise programs such as chair aerobics or yoga and exercise training with a training frequency less than 2 days/week were also not included. Since Tai Chi is a weight-bearing exercise, beneficial effects may be expected, which validate its eligibility as a training program.

Outcome measures for this review were defined as BMD (grams per square centimeter) at the lumbar spine and femoral neck measured by radiographic techniques (single photon absorptiometry (SPA), dual photon absorptiometry (DPA) or dual X-ray absorptiometry (DXA)) with standard deviations (SD).

Data were collected at baseline and the most distal data collection point available when reports presented outcomes at different intervals (unless it would severely reduce the sample size) given the higher possibility of more marked results (changes).

For studies that met our inclusion criteria but did not provide appropriate information on changes in BMD, we personally tried to contact the authors to retrieve such information. Studies were excluded when authors did not respond or the data were no longer available.

Search methods for identification of studies

A computerized literature search of the MEDLINE, PubMed, Academic Search Complete, CINAHL plus, Scopus, Sport Discus and Web of Knowledge databases was conducted from their inception to February 2011 by one reviewer (EAM). The text words, key words and subject headings used were (exercise* OR "physical activity" OR training) AND ("bone density" OR "bone mineral density"). No limits of the search (including language restrictions, human studies or age) were used at this stage. The reference lists of all identified retrieved studies and some review articles were carefully checked aiming to identify potential interesting studies not found in the primary electronic search. In addition, hand searching of key peer-reviewed journals (*Bone*, *Calcified Tissue International*, *Journal of Bone and Mineral Metabolism*, *Journal of Bone and Mineral Research*, *Osteoporosis International*, and *Medicine and Science in Sports and Exercise*) was also performed to identify possible missed RCTs in database searches. Citations were entered into the reference management software EndNote, version X2 (Thomson Reuters, Carlsbad, CA, USA).

Data collection and analysis

Selection of studies

The titles and abstracts of studies identified in the computerized searches (7727 reports) were examined by two authors (EAM and JM) in order to remove obvious irrelevant reports. An over-inclusive policy was adopted at this stage, which implied that in the absence of any information to the contrary, each article was forwarded to the next stage of the screening process. Criteria for inclusion were titles, abstracts, and/or articles that did not meet the exclusion criteria. Full-text of 113 reports was closely screened to identify those studies complying with the eligibility criteria by two authors independently (EAM and JC). In the case of disagreements, decisions were made by joint consensus between the authors.

Multiple publication bias (trials reporting BMD data for the same participants in more than one publication) was examined by analyzing each study to ensure that data from only one of the articles were included to avoid double-counting participants.

Clarification of data and missing results was obtained by correspondence with authors. Blinding of the investigators to the name of the author, institutional affiliation, journal of publication and study results were not performed because it has been shown that these procedures have no clinically or statistically significant effect on results (Berlin 1997).

Data extraction and management

Data extraction was completed using a pilot-tested and revised coding frame to record information on a range of details. The major categories of variables coded included source characteristics (e.g. country, publication year and presence of funding), study design (e.g. number of allocated participants and number of participants followed up, follow-up length, attrition, compliance, exercise supervision, any adjuvant pharmacological or nutritional therapy affecting bone and intent-to-treat analysis), sample characteristics (e.g. gender, age, health and/or functional status), intervention (e.g. type, frequency and duration of the exercise interventions), scanning technique and outcome measures (BMD values with standard deviations). All data were coded and reviewed for accuracy and consistency by the first author.

Assessment of risk of bias in included studies

The methodological quality of the studies was assessed with the Cochrane risk of bias assessment tool (Higgins and Green 2009), which addresses six specific domains, namely sequence generation, allocation concealment, blinding, incomplete outcome data, selective outcome reporting and other potential sources of systematic bias. Each domain includes one or more specific entries in a 'Risk of bias' table. Within each entry, the first part of the tool involves the description of what was reported to have happened in the study. The second part of the tool involves assigning a judgment related to the risk of bias for that entry, such that a judgment of 'Yes' indicates low risk of bias, 'No' indicates high risk of bias and 'Unclear' indicates unclear or unknown risk of bias. In cases of possible disagreement between the reviewers (EAM and JM), after reanalysis of the article a joint decision was made. Particularly, we were concerned in about whether intention-to-treat (ITT) rather than a per-protocol approach was used in analyzing the data of original articles and if attrition and exclusions from the analysis were reported (item 4). Moreover, as the capacity to detect an osteogenic response is closely associated with the dose of stimuli, the sample size and the follow-up length (item 6), these issues were considered as other potential sources of bias, which were not addressed in the other domains in the tool. Thus, based in this empirical evidence of bias, incomplete outcome data and other source of bias were the two domains with higher relevance in the present review. A risk of bias summary graph was generated according to recommendations by the Cochrane guidelines (Higgins and Green 2009).

Continuous data

Given that our outcome (BMD) was reported on a meaningful scale and all studies in the analysis used the same scale (grams per square centimeter), the effect size (ES) was computed directly on the raw (unstandardized) difference in means (D). We combined trials reporting mean final values with trials reporting mean changes in the same meta-analysis. When studies reported both final and change we used the change score to compute D. Thus, D was calculated from studies that used independent groups as the difference between treatment group vs. comparison group final value means or the difference between absolute change-from-baseline values. A positive D reflects more favorable outcome scores for treatment groups than for the comparison group. Due to variable reporting from the data summaries presented in the individual studies, standard deviation was obtained from the standard error (SE) by multiplying SE by the square root of the sample size; confidence intervals for means were also used to calculate standard deviations using

$$\mathrm{SD} = \sqrt{N} \times (\text{upper limit} - \text{lower limit})/95\%\,\mathrm{CI}^*$$

*calculated using a value from a t distribution (with degrees of freedom equal to the group sample size minus 1) as explained with detail in the *Cochrane Reviewers' Handbook* (Higgins and Green 2009).

Studies with multiple treatment groups

For trials that randomized participants to one of several relevant experimental intervention groups with a common control group (multi-arm studies), each pair-wise comparison was included separately, although with the *shared* control group divided into two groups with a smaller sample size. Nevertheless, the means and standard deviations were preserved unchanged. This process partially overcomes the unit-of-analysis error and ensures that control participants are not counted more than once within the meta-analysis, representing a practical method of performing investigation of heterogeneity (Higgins and Green 2009).

Dealing with missing data

Where final values were not available from the original article or author, post-means were extracted from mean percent change, and SD were imputed using the pre-training SD as it is reasonable to assume that the intervention does not alter the variability of the outcome measure (Higgins and Green 2009). Reported results regarding missing individual participants were not imputed, and thus we included data for only those participants whose results were known.

Assessment of heterogeneity

In order to express informative heterogeneity indices, a measure of both the magnitude and of uncertainty were presented. Magnitude was represented by both the degree of true variation (the between-studies variance) on the scale of the effect measure (T^2) and the degree of inconsistency (I^2, *I*-squared, the ratio of true heterogeneity to total observed variation). Uncertainty over whether apparent heterogeneity is genuine was expressed using the *p*-value (<0.10 was considered significant (Higgins and Green 2009) since the *Q* statistic tends to suffer from low differential power) for *Q* statistic (a measure of weighted squared deviations) and using confidence intervals for I^2.

The *Q* statistic has the advantage of being expressed on a standard scale and is sensitive to the number of studies; T^2 is independent of the number of studies but is expressed on the original metric. Finally, the I^2 statistic is not directly affected by the number of studies in the analysis and expresses the result as a ratio (proportion of the observed variance reflects real differences in effect size). Generally, values of 25%, 50% and 75% are considered to be indicative of small, moderate and large amounts of inconsistency, respectively (Higgins and Green 2009).

Assessment of reporting biases

It is possible that the studies included in our meta-analysis may overestimate the true effect size as they are based on a biased sample of the target population of studies. Thus, to examine if there is evidence of any bias, we tested the relationship between standard error on the vertical axis and effect size on the horizontal axis (funnel plot) using the Egger's linear regression method (Sterne et al. 2000; Egger et al. 1997).

Another approach to deal with publication bias was applying the trim-and-fill procedure of Duval and Tweedie (Duval and Tweedie 2000). This approach essentially addresses the question, "*What is our best estimate of the unbiased effect size?*" (p. 289) (Borenstein et al. 2009). Trim-and-fill is an iterative non-parametric method used to investigate the number of "missing" studies in a meta-analysis, as indicated by funnel plot asymmetry, and calculates an adjusted pooled estimate with the addition of those "missing" studies (Duval and Tweedie 2000). The probability level of $p<0.05$ was used to indicate statistical significance.

All the approaches used (funnel plot, the regression test and trim-and-fill) are based on a model which assumes that if the effect size is higher in the smaller studies, then publication bias is the reason. In addition, the procedures to address publication bias are subject to a number of caveats; mostly they tend to have lower power, and trim-and-fill approach can be influenced by one or two aberrant studies (Borenstein et al. 2009).

Data synthesis

Outcomes were analyzed as continuous using a random-effects meta-analysis as studies apparently differ in the mixes of participants and in the implementations of interventions, and thus there may be different effect sizes underlying different studies. We calculated a weighted mean, where the weight assigned to each study is the inverse of that study's variance, and the variance includes the original (within-studies) variance (*V*) plus the estimate of the between-studies variance, T^2 (tau-squared). T^2 was estimated

using the method of moments (or the DerSimonian and Laird). Study weights were assigned with the goal of minimizing both sources of variance, which are more balanced under the random-effects model than under the fixed-effect model. Additionally, large studies are assigned less relative weight, and small studies are assigned more relative weight as compared with the fixed-effect model.

Thus, the computed summary effect is our estimate of the mean of the distribution of all relevant true effects, and the null hypothesis is that the mean of these effects is zero. To establish the statistical significance of our results 95% confidence intervals (CIs) were used. All analyses were conducted with Comprehensive Meta Analysis software version 2.2.048 (Biostat, Englewood, NJ, USA).

Sensitivity analysis

In order to test the impact of alternative decisions or ranges of values for decisions that were arbitrary or unclear during the process of undertaking the present systematic review (which is inevitable), a sensitivity analysis was performed to assess the following decisions: Should analyses be based on change scores and final values simultaneously? Should both genders be combined? What range of dose (training frequency) should be included? Should small sample sizes be included? Should studies which add supplements of calcium and/or vitamin D be combined with no supplementation studies? Can different impact loading exercise protocols be combined in a single summary effect? Exercise protocols were categorized according to the impact classifications described by Nikander et al. (2005) and acceleration forces observed by Vainionpaa et al. (2006), and previously described by Martyn-St James and Carroll (2009). Exercise protocols exclusively based on resistance training were labeled as a new impact group.

Results

Description of studies

Results of the search

The initial database search yielded 18845 possible articles which were screened against the inclusion and exclusion criteria (Fig. 1). Of these publications, 7727 abstracts were identified without duplicates, and 7614 were rejected at the title and abstract stage. One hundred and thirteen studies were identified for potential inclusion and full-text reports were analyzed, and 19 were entered in the analysis (Brooke-Wavell et al. 1997; Chuin et al. 2009; Englund et al. 2005; Jessup et al. 2003; Kemmler et al. 2010; Korpelainen et al. 2006; Lau et al. 1992; Lord et al. 1996; Marques et al. 2011a, b; Nichols et al. 1995; Park et al. 2008; Rhodes et al. 2000; Taaffe et al. 1999; Villareal et al. 2004; Vincent and Braith 2002; von Stengel et al. 2011a, b; Woo et al. 2007).

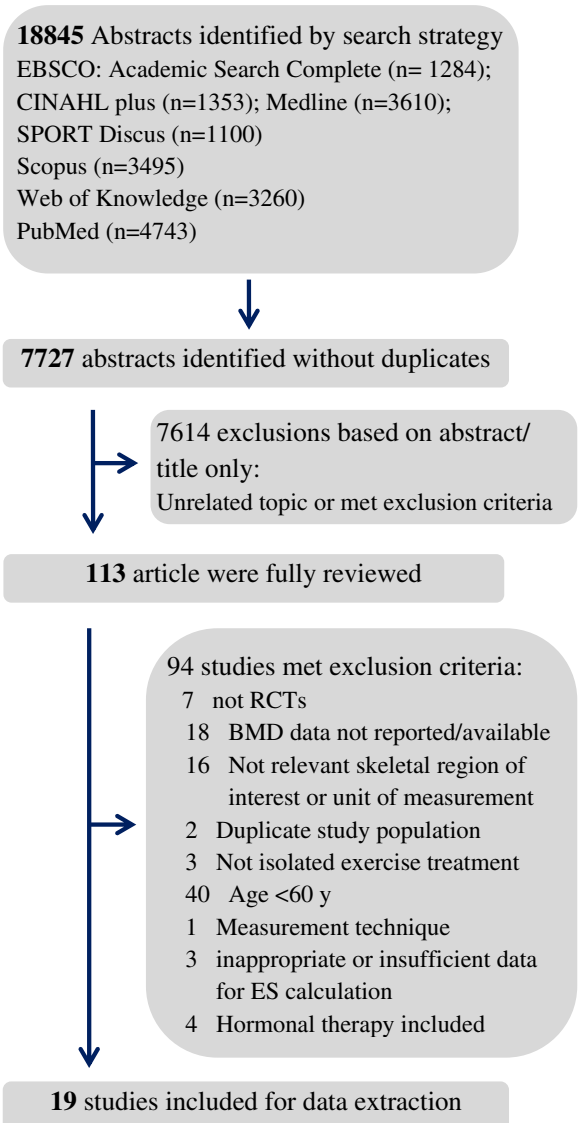

Fig. 1 Flow chart depicting the trial flow for selection of randomized controlled trials (RCTs) to be included

Included studies

Study characteristics are shown in Table 1. Taking the 19 studies together, results were reported from 26 interventions, and the overall sample size was 1577 participants. The sample sizes of the individual studies varied. While small sample sizes had approximately 20 subjects, the large sample sizes ranged between 112 and 227 subjects. The median value for mean age was 69 years, and the mean age ranged from 65 to 83 years. Fifteen studies (Brooke-Wavell et al. 1997; Chuin et al. 2009; Englund et al. 2005; Jessup et al. 2003; Kemmler et al. 2010; Korpelainen et al. 2006; Lau et al. 1992; Lord et al. 1996; Marques et al. 2011a, b; Nichols et al. 1995; Park et al. 2008; Rhodes et al. 2000; von Stengel et al. 2011a, b) were focused exclusively on women (n=1339, 85%). Participants were mostly community-dwelling and leaving independently, excepting one study which included subjects with mild-to-moderate physical frailty (Villareal et al. 2004) and another study that recruited subjects from a hostel for elderly subjects (Lau et al. 1992). One study (Korpelainen et al. 2006) included women with low BMD (hip BMD 2 SD below the reference value). Minority inclusion was infrequently reported: four studies included only Caucasian subjects (Chuin et al. 2009; Jessup et al. 2003; Marques et al. 2011a, b), 3 included Asians (Lau et al. 1992; Park et al. 2008; Woo et al. 2007) and only one study included a small proportion (≈15%) of non-Caucasians (Villareal et al. 2004). Three trials (Lau et al. 1992; Park et al. 2008; Woo et al. 2007) were carried out in Asia, while 8 (Brooke-Wavell et al. 1997; Englund et al. 2005; Kemmler et al. 2010; Korpelainen et al. 2006; Marques et al. 2011a, b; von Stengel et al. 2011a, b) were in Europe and 8 (Chuin et al. 2009; Jessup et al. 2003; Lord et al. 1996; Nichols et al. 1995; Rhodes et al. 2000; Taaffe et al. 1999; Villareal et al. 2004; Vincent and Braith 2002) in North America. A few reports appeared before the year of 2000, and 12 studies were published between 2002 and 2011. Attrition was typically small (mean=14.2%), though four studies experienced more pronounced losses (around 25%). Attrition was similar between treatment and control groups. Most studies measured BMD outcomes immediately after completing the intervention, and only one report (Lord et al. 1996) measured the outcomes within 3 weeks after intervention. Six studies (Marques et al. 2011b; Taaffe et al. 1999; Vincent and Braith 2002; von Stengel et al. 2011a, b; Woo et al. 2007) included multiple treatment groups which enabled us to calculate an ES for 12-treatment-group vs. control-group comparisons. For those studies in which dropout information was discriminated, 11 used a per-protocol approach to analyze their data (Brooke-Wavell et al. 1997; Englund et al. 2005; Jessup et al. 2003; Lau et al. 1992; Lord et al. 1996; Nichols et al. 1995; Rhodes et al. 2000; Taaffe et al. 1999; Villareal et al. 2004; Vincent and Braith 2002; Woo et al. 2007), six used the ITT approach (Kemmler et al. 2010; Korpelainen et al. 2006; Marques et al. 2011b; Park et al. 2008; von Stengel et al. 2011a, b) and one study used both approaches for data analysis (Marques et al. 2011a). One study did not report having any dropouts (Chuin et al. 2009).

With respect to intervention characteristics, the most common training modalities were either resistance exercise or a combination of a resistance/strength exercise component with endurance and/or balance exercises. Thus, 15 interventions (62.5%) included a strength or resistance exercise component (Chuin et al. 2009; Englund et al. 2005; Jessup et al. 2003; Kemmler et al. 2010; Lord et al. 1996; Marques et al. 2011a, b; Nichols et al. 1995; Park et al. 2008; Rhodes et al. 2000; Taaffe et al. 1999; Villareal et al. 2004; Vincent and Braith 2002; von Stengel et al. 2011a; Woo et al. 2007). Four studies used a combination of strength, aerobic (odd-impact loading) and balance exercises (Englund et al. 2005; Kemmler et al. 2010; Lord et al. 1996; von Stengel et al. 2011a), two studies combined strength, weight-bearing and balance exercises (Marques et al. 2011a; Park et al. 2008) and two studies used aerobic activities (including walking) as the primary intervention (Brooke-Wavell et al. 1997; Marques et al. 2011b). Other two studies used weight-bearing activities (such as jumping or stepping up and down a block) as the primary training modality (Korpelainen et al. 2006; Lau et al. 1992). One intervention exclusively consisted of Tai Chi sessions (Woo et al. 2007), and another one included only whole body vibration training (von Stengel et al. 2011b). Seven interventions were entirely composed of strength or resistance exercise training (Chuin et al. 2009; Marques et al. 2011b; Nichols et al. 1995; Rhodes et al. 2000; Taaffe et al. 1999; Vincent and Braith 2002; Woo et al. 2007), and one (von Stengel et al. 2011a) incorporated whole body vibration combined with conventional training (including strength,

Table 1 Characteristics of included studies

Study	Country	Subjects[a]	Exercise intervention[b]	Supplementation; supervision	Dropout; compliance	BMD outcomes and device	Comparison group[c]
Brooke-Wavell et al. 1997	UK	78 subjects assigned into an EG (n=38, mean age=64.9 y) and a CG (n=40, mean age=64.2 y); 100% female	52 wk of training that consisted of self-monitored walking 3.5 times per week for 14.8 min/day for the first 12 wk, followed by 20.4 min/day of walking, 4.8 days/wk, for the subsequent 40 wk	No additional suppl; supervision: no	6%; not reported	Lumbar spine (L2–L4) and femoral neck assessed by DXA (Lunar)	Non-exercise control (9 women take up swimming (2 days/wk, 20 min/session)[c]
Chuin et al. 2009	Canada	18 subjects assigned into an EG (n=11, mean age=65.4 y) and a CG (n=7, mean age=67.4 y); 100% female	24 wk of RT performed 3 days/wk for 60 min/session. Exercise sessions consisted of 15 min warm-up and 3 sets of 8 rep at 80% of 1 RM (45 min), focused on the large and small muscle groups of the upper and lower body	No additional suppl; supervision: yes	0%; >91.7%	Lumbar spine (L2–L4) and femoral neck assessed by DXA (Lunar)	Non-exercise placebo
Englund et al. 2005	Sweden	40 subjects assigned into an EG (n=21, mean age=72.8 y) and a CG (n=19, mean age=73.2 y); 100% female	52 wk of training performed 2 days/wk for 50 min/session. Exercise sessions consisted of 10 min warm-up, 10 min aerobic, 12 min strength, 5 min balance and coordination, 11 min cool-down/stretching/relaxation	No additional suppl; supervision: yes	18.8%; 67%	Lumbar spine (L2–L4) and femoral neck assessed by DXA (Lunar)	Non-exercise control
Jessup et al. 2003	USA	18 subjects assigned into an EG (n=9, mean age=69.1 y) and a CG (n=9, mean age=69.4 y); 100% female	32 wk of training performed 3 days/wk for 60–90 min/session. Exercise sessions consisted of 5 min warm-up, strength exercises; load-bearing walking and stair climbing for 35–40 min and balance exercises while wearing weighted vests (carry up to 10% of the participant's body weight), 5 min cool-down	1,000/day of calcium and 400 IU/day of vitamin D; supervision: yes	10%; not reported	Lumbar spine (L2–L4) and femoral neck assessed by DXA (Norland)	Non-exercise control
Kemmler et al. 2010	Germany	227 subjects assigned into an EG (n=115, mean age=68.9 y) and a CG (n=112, mean age=69.2 y); 100% female	18 months of training performed 2 days/wk for 60 min/session and 2 days/wk of home sessions (20 min each). Exercise sessions consisted of warm-up/aerobic dance (20 min at 70%–80% maximum HR), balance training (5 min), functional gymnastics, strength exercise for the upper body and unilateral dynamic weight-bearing leg exercises; home exercises emphasized strength and flexibility exercises	Calcium (1,500 mg/day) and vitamin D 500 UI/day; supervision: yes (group sessions)	7.7%; 76.3% (group sessions) and 42.2% (home sessions)	Lumbar spine (L1–L4) and femoral neck assessed by DXA (Hologic)	Low-intensity wellness program (designed not to cause physical adaptations)[c]
Korpelainen et al. 2006	Finland	160 subjects assigned into an EG (n=84, mean age=72.9 y) and a CG (n=76, mean age=72.8 y);	30 months of training that included a 60-min supervise group session (1 day/wk) for a 6-month period each year and home sessions (20 min each) usually performed	No additional suppl; supervision: yes (group sessions)	16.9%; 75% (group sessions)	Femoral neck assessed by DXA (Lunar)	Non-exercise control

		100% female	3 days/wk. Group sessions included 15 min warm-up and 45 min devoted to jumping and balance exercises including walking, knee bends, leg lifts, heel rises and drops, dancing, stamping, stair climbing and stepping up and down from benches; home exercises were similar to those in the supervised sessions				
Lau et al. 1992	China	23 subjects assigned into an EG (n=11, mean age=79 y) and a CG (n=12, mean age=75 y); 100% female	40 wk of training performed 4 days/wk. Exercise sessions consisted of step up and down a block (9 inches/23 cm) 100 times and 15 min of upper trunk exercises while standing	No additional suppl; supervision: yes	16.7%; not reported	Lumbar spine (L2–L4) and femoral neck assessed by DXA (Norland)	Non-exercise control
Lord et al. 1996	Australia	138 subjects assigned into an EG (n=68, mean age=71.7 y) and a CG (n=70, mean age=71.5 y); 100% female	42 wk of training performed 2 days/wk for 6 min/session. Exercise sessions consisted of 5 min warm-up; 35 min aerobic, balance, coordination and strengthening exercises; 15 min stretching; 5–10 min cool-down period	No additional suppl; supervision: yes	22.9%; 72.9%	Lumbar spine (L2–L4) and femoral neck assessed by DXA (Lunar)	Non-exercise control
Marques et al. 2011a	Portugal	60 subjects assigned into an EG (n=30, mean age=70.1 y) and a CG (n=30, mean age=68.2 y); 100% female	32 wk of training performed 2 days/wk for 60 min/session. Exercise sessions consisted of 10 min warm-up; 15 min weight-bearing activities, 10 min muscular endurance, 10 min balance training, 10 min agility training, 5 min stretching	No additional suppl; supervision: yes	18.3%; 68.3%	Lumbar spine (L1–L4) and femoral neck assessed by DXA (Hologic)	Non-exercise control
Marques et al. 2011b	Portugal 2011	71 subjects assigned into an REG (n=23, mean age=67.3 y), an AEG (n=24, mean age 70.3 y) and a CG (n=24, mean age=67.9 y); 100% female	32 wk of training performed 3 days/wk for 60 min/session.RE sessions consisted of 8–10 min warm-up, 30–40 min specific resistance training which included 2 sets of 6–8 rep at 75–80% of 1 RM, focused on quadriceps, hamstrings, gluteus, trunk, arms and abdominal wall muscle groups; 5–10 min cool-down period. AE sessions 10–15 min warm-up, 35–40 min of dynamic aerobic activities (65–85% heart rate reserve), strength exercises (first 6 weeks), 10 min cool-down period	No additional suppl; supervision: yes	23.9%; 78.4% (RE) and 77.7%(AE)	Lumbar spine (L1–L4) and femoral neck assessed by DXA (Hologic)	Non-exercise control
Nichols et al. 1995	USA	28 subjects assigned into an EG (n=14, mean age=67.8 y) and a CG (n=14, mean age=65.2 y); 100% female	24 wk of isotonic weight training performed 3 days/wk. Exercise sessions consisted of 1 set of 10–12 rep at 50% of 1 RM and progressed to 3 sets at 80% of 1 RM	Calcium intake 800 mg/day; supervision: yes	17.6%; 86.8%	Lumbar spine (L2–L4) and femoral neck assessed by DXA (Lunar)	Non-exercise control
Park et al. 2008	Japan	50 subjects assigned into an EG (n=25, mean age=68.3 y)	48 wk of training performed 3 days/wk for 60 min/session. Exercise sessions consisted of	No additional suppl; supervision: yes	10%; not reported	Lumbar spine (L2–L4) and femoral neck	Non-exercise control

Table 1 (continued)

Study	Country	Subjects[a]	Exercise intervention[b]	Supplementation; supervision	Dropout; compliance	BMD outcomes and device	Comparison group[c]
		and a CG (*n*=25, mean age=68.4 y); 100% female	stretching, strength, weight-bearing (at an intensity above 65%–5% of the maximal HR), balance and posture correction training			assessed by DXA (Lunar)	
Rhodes et al. 2000	Canada	44 subjects assigned into an EG (*n*=20, mean age=68.8 y) and a CG (*n*=18, mean age=68.2 y); 100% female	1 year of RT performed 3/wk for 60 min/session. Exercise sessions consisted of 20 min warm-up and 3 sets of 8 rep at 75% of 1 RM performed in circuit, focused on the large muscle groups of the upper and lower body	No additional suppl; supervision: yes	13.6%; ≈85%	Lumbar spine (L2–L4) and femoral neck assessed by DXA (Lunar)	Non-exercise control
von Stengel et al. 2011b	Germany	96 subjects assigned into a VVTG (*n*=34, mean age=68.1 y), an RVTG (*n*=29, mean age=67.9 y) and a CG (*n*=33, mean age=67.6 y); 100% female	1 year of whole body vibration training performed 3 days/wk for 15 min/session. Exercise sessions consisted of 7 one-legged or two-legged dynamic leg strengthening exercises, performed on the plates in standing position; VVT G vibrated at a frequency of 35 Hz and the RVTG at 12.5 Hz	Calcium (1,200 mg/day) and vitamin D (800 UI/day); supervision: no[c]	11.1%; 73% (VVTG) and 68% (RVTG)	Lumbar spine (L1–L4) and femoral neck assessed by DXA (Hologic)	Low-intensity wellness program (designed to avoid impact on our primary endpoints)[4]
Taaffe et al. 1999	USA	46 subjects assigned into an EG 1 (*n*=12, mean age=69.4 y), EG 3 (*n*=11, mean age=71.0 y) and a CG (*n*=12, mean age=68.9 y); 36% female	24 wk of RT performed 2 days/wk (EG1) and 3 days/wk (EG2). Exercise sessions consisted of warm-up, 3 sets of 8 rep at 80% of 1 RM, focused on the large muscle groups of the upper and lower body, and cool-down (stretching) period	No additional suppl; supervision: yes	10.3%; 99% and 97%	Lumbar spine (L2–L4) assessed by DXA (Hologic)	Non-exercise control
Villareal et al. 2004	USA	112 subjects assigned into an EG (*n*=65, mean age=83 y) and a CG (*n*=47, mean age=83 y); 53.6% female	36 wk of training performed 3 days/wk for 90–120 min/session. Exercise sessions consisted of successive phases of physical therapy, resistance (progressed to 3 sets, 8–12 rep at 85–90% of 1 RM) and endurance exercises (progressed to 4×5 min at 85–90% of peak HR)	Calcium and vitamin D to adjust intake to 1,200 mg/d and 800 U/d; supervision: yes	26.9%; 73.3% (exercise) and 96.7% (control)	Lumbar spine (L2–L4) and femoral neck assessed by DXA (Hologic)	Home-exercise group (focused primarily on flexibility)[c]
Vincent and Braith 2002	USA	62 subjects assigned into an LEXG (*n*=24, mean	24 wk of RT performed 3 days/wk for 30 min/session. Exercise sessions included warm-up, 13	No additional suppl; supervision: yes	26.2%; >85% (LEX and HEX)	Lumbar spine (L2–L4) and femoral neck	Non-exercise control

		age=67.6 y), HEXG (*n*=22, mean age=66.6 y) and a CG (*n*=16, mean age=71 y); not reported	exercises on resistance machines and cool-down. LEX sessions consisted 1 set of 13 rep at 50% of 1 RM and HEX sessions consisted of 1 set of 8 rep at 75% of 1 RM			assessed by DXA (Lunar)	
von Stengel et al. 2011a	Germany	141 subjects assigned into an EG (*n*=47, mean age=68.6 y), EG plus vibration group (EVG, *n*=46, mean age=68.8 y) and a CG (*n*=48, mean age=68.1 y); 100% female	72 wk of training performed 2 days/wk for 60 min/session and 2 days/wk of home sessions (15–20 min each). Controlled exercise sessions consisted of dancing aerobics, balance training, functional gymnastics and dynamic leg-strength training on vibration plates (without vibration). EVG performed an identical exercise regimen with vibration (25–35 Hz) during the leg-strengthening sequence. Home training session included some strength and stretching exercises of the joint session	Calcium and vitamin D to adjust intake to 1,500 mg/d and 400 IE/day; supervision: yes (joint sessions)	10.6%; 75%/43% (joint sessions/home sessions EG) and 80%/45% (joint sessions/home sessions EVG)	Lumbar spine (L1–L4) assessed by DXA (Hologic)	Low-intensity wellness program[4]
Woo et al. 2007	China	176 subjects assigned into a Tai Chi group (*n*=28 W, mean age=69.7 y/30 M, mean age=68.2 y), a REG (*n*=29 W, mean age=69.6 y/30 M, mean age=68.7 y) and a CG (*n*=30 W, mean age=69.6 y/29 M, mean age=68.1 y); 50% female	52 wk of training performed 3 days/wk. Tai Chi sessions used the Yang style with 24 forms. RE sessions included 6 exercises repeated 30 times using theraband of medium strength	No additional suppl; supervision: no mention	2.2%; 81% (tai chi) and 76.6% (RE)	Lumbar spine (L2–L4) and femoral neck assessed by DXA (Hologic)	Non-exercise control

EG exercise group, *CG* control group, *RT* resistance training, *rep* repetitions, *RM* one repetition maximum, *LEXG* low-intensity exercise group, *HEXG* high-intensity exercise group, *REG* resistance exercise group, *AEG* aerobic exercise group, *VVTG* vertical vibration training group, *RVT* rotational vibration training group, *W* women, *M* men, *suppl* supplementation, *d* day(s), *wk* week(s), *y* year(s)

[a] Number of participants included in the analysis (final sample size)

[b] Exercise protocol, frequency, intensity and duration

[c] Only the first 3 sessions, and every 6 weeks an instructor controlled if the exercises were still being executed properly

[4] Intervention that would not be expected to improve our primary outcome measures

endurance, balance and functional exercises). The remaining two interventions used a combination of resistance exercise with endurance (Villareal et al. 2004) or with weight-bearing exercises (Jessup et al. 2003). Resistance exercise intensity ranged between 50 and 90% of 1 repetition maximum (1-RM). Aerobic intensity ranged from 65 to 90% of sub-maximum heart rate.

Most studies were center-based: they were delivered in places such as a community center, a training facility or a gymnasium. In three studies (Kemmler et al. 2010; Korpelainen et al. 2006; von Stengel et al. 2011a), participants received interventions both in a group setting at a center and as individuals in their own homes. One intervention consisted of self-monitored walking (Brooke-Wavell et al. 1997). Details regarding supervision of the exercise sessions, apart from home sessions which were not supervised, were not reported in one trial (Woo et al. 2007), and one study reported that all walking sessions were unsupervised (Brooke-Wavell et al. 1997). Compliance with the prescribed exercise (group) interventions measured as a percentage of attended sessions ranged from 67% to 99%, and the median attendance was 77%. Four studies did not report attendance rate (Jessup et al. 2003; Lau et al. 1992; Park et al. 2008). In general interventions were delivered three to four times per week, and exercise sessions lasted at least 60 min. Home sessions tended to be shorter (lasting around 20 min). Session duration was not specified in five of the included interventions (Nichols et al. 1995; Taaffe et al. 1999; Woo et al. 2007). The length from baseline to post-intervention test ranged from 6 (24 weeks) to 30 months, whereas 13 interventions (Brooke-Wavell et al. 1997; Chuin et al. 2009; Kemmler et al. 2010; Korpelainen et al. 2006; Lau et al. 1992; Lord et al. 1996; Park et al. 2008; Rhodes et al. 2000; von Stengel et al. 2011a, b; Woo et al. 2007) lasted 40 weeks or more. Five interventions from five studies (Jessup et al. 2003; Kemmler et al. 2010; Villareal et al. 2004; von Stengel et al. 2011a, b) increased daily calcium and vitamin D intake levels of all participants (to adjust calcium intake up to 1000–1500 mg and vitamin D up to 400–800 IU) by means of supplementation during the intervention. One study (Nichols et al. 1995) only increased daily calcium intake level to 800 mg via dairy products or calcium supplement.

BMD outcomes were measured before and after intervention in each individual study by the same radiographic techniques (DXA), but measurements were taken using DXA devices from three different manufacturers (Norland, Cooper Surgical, Trumbull, CN, USA; Lunar, GE Medical Systems, Madison, WI, USA and Hologic Bedford, MA, USA). Four trials reported ongoing BMD assessment at more than two time-points (Korpelainen et al. 2006; Nichols et al. 1995; Villareal et al. 2004; Woo et al. 2007). BMD at lumbar spine was assessed in 17 trials (Brooke-Wavell et al. 1997; Chuin et al. 2009; Englund et al. 2005; Jessup et al. 2003; Kemmler et al. 2010; Lau et al. 1992; Lord et al. 1996; Marques et al. 2011a; Nichols et al. 1995; Park et al. 2008; Rhodes et al. 2000; Taaffe et al. 1999; Villareal et al. 2004; Vincent and Braith 2002; von Stengel et al. 2011a, b; Woo et al. 2007), and femoral neck BMD was also assessed in 16 trials (Brooke-Wavell et al. 1997; Chuin et al. 2009; Englund et al. 2005; Jessup et al. 2003; Kemmler et al. 2010; Korpelainen et al. 2006; Lau et al. 1992; Lord et al. 1996; Marques et al. 2011a, b; Nichols et al. 1995; Park et al. 2008; Rhodes et al. 2000; von Stengel et al. 2010b; Villareal et al. 2004; Vincent and Braith 2002); thus 14 trials included both bone site measures (Brooke-Wavell et al. 1997; Chuin et al. 2009; Englund et al. 2005; Jessup et al. 2003; Kemmler et al. 2010; Lau et al. 1992; Lord et al. 1996; Marques et al. 2011a; Nichols et al. 1995; Park et al. 2008; Rhodes et al. 2000; von Stengel et al. 2011b; Villareal et al. 2004; Vincent and Braith 2002). Three trials did not focus on femoral neck (Taaffe et al. 1999; von Stengel et al. 2011a; Woo et al. 2007) and 2 trials on lumbar spine site (Korpelainen et al. 2006; Marques et al. 2011b).

Mean final values in BMD along with SDs were available for nine studies (Chuin et al. 2009; Englund et al. 2005; Jessup et al. 2003; Lord et al. 1996; Marques et al. 2011a, b; Park et al. 2008; Rhodes et al. 2000; Vincent and Braith 2002). Absolute change values in BMD at follow-up along with SDs were available for four studies (Brooke-Wavell et al. 1997; Kemmler et al. 2010; von Stengel et al. 2011a, b). Final means were extracted from mean percent change for two studies (Lau et al. 1992; Woo et al. 2007). SDs were imputed using the pre-training SD for one study (Woo et al. 2007), while the SDs of another study (Lau et al. 1992) were obtained from CI. Two studies (Nichols et al. 1995; Taaffe et al. 1999) reported final means and SE, and one study (Korpelainen et al. 2006) reported final means and

CI; thus SDs were obtained from SE and CI, respectively.

Excluded studies

Ninety-four articles were excluded because of no relevant skeletal region of interest or unit of measurement (n=16), age blow 60 years (n=40), no RCT (n=7), duplicate study population (n=2), no BMD data reported or available (n=18), hormonal therapy included (n=4), no isolated exercise treatment (n=3), no extractable data (n=3) and other measurement technique (n=1).

Risk of bias in included studies

Risk of bias is summarized in Fig. 2, which illustrates the methodological quality summary. Judgments ('yes', 'no', 'unclear') for each domain across all the included studies are shown, demonstrating an overall low across-studies potential risk of bias. Thus, most trials had low risk of bias across the six domains. Risk of bias for the domain 'other sources of bias' is 'high' only for the question-based entry 'small sample size', and the entry 'exercise duration' has a moderate risk of bias as 10 trials were sustained for less than 12 months.

Effects of interventions

Data on lumbar spine BMD were available for 22 study group comparisons of exercise interventions vs. control from 17 of the included trials. A total of 769 exercise intervention participants and 571 controls were included. Exercise interventions resulted in a small increase in BMD at this site of 0.011 g/cm^2 (WMD, 95% CI 0.003 to 0.020; p=0.007). Heterogeneity was statistically significant in this subset of studies (Q=44.0, df=21, p=0.002); thus studies do not share a common effect size. Our estimate for the SD of the true effect sizes (T) was 0.011. Therefore, considering that our summary effect was 0.011, we expected that some 95% of the true effects will fall in the approximate range of −0.011 to 0.033. In addition, the degree of inconsistency was moderate (I^2=52%, 95% CI 22–71%), which indicates that a considerable amount of the variability across studies was due to heterogeneity rather than chance.

The 16 trials assessing femoral neck BMD provided 19 study group comparisons, including 651 subjects in the intervention group and 541 subjects in the control group. The combined WMD in BMD was 0.016 g/cm^2 (95% CI 0.005 to 0.027; p=0.004). Heterogeneity was statistically significant in this analysis (Q=49.4, df=18, p<0.001). The SD of the summary effect (T) was 0.015; thus most effects will fall in the range of −0.013 to 0.045. Moreover a moderate proportion of the observed variance was real (I^2=64%, 95% CI 40–78%). Figures 3 and 4 show the results from meta-analysis of all included RCTs. Table 2 lists results from meta-analyses and all sensitivity analyses.

Sensitivity analysis including only final values generally did not show any divergence from the heterogeneity evident in the primary analyses including final and change values at lumbar spine and femoral neck (I^2≈55%). The WMD in BMD at lumbar spine was 0.016 g/cm^2 (95% CI −0.004 to 0.036; p=0.111), and an increase in BMD of 0.020 g/cm^2 (95% CI 0.003 to 0.037; p=0.023) was observed at femoral neck. Under the random-effects model, the WMD in BMD at both sites were nearly identical for all trials and trials including only females (0.011 vs. 0.012 for lumbar spine and 0.016 vs. 0.014), and heterogeneity in both sites (I^2≈65%) was not divergent from the analysis with all trials. Similarly, sensitivity analyses for the effects of high training frequency (≥3 days/week) did not show divergent results from the primary meta-analysis, with similar moderate heterogeneity in both analyses (I^2≈60%). No significant effects in BMD were evident at lumbar spine when subgroup analysis excluded trials including calcium and/or vitamin D supplementation. Although a more pronounced overall effect was observed for femoral neck (WMD=0.022 g/cm^2), the analysis for this subgroup of trials was heterogeneous (I^2=69%). Treatment effects at both lumbar spine and femoral neck were significant and more robust when the analysis only included trials with a total sample of more than 50 participants, but I^2 value for femoral neck analysis increased to 79%.

The subgroup analysis of trials evaluating protocols of combined loading protocols that incorporated impact activity with resistance exercises was consistent in showing positive effects of this type of exercise on BMD at the lumbar spine (I^2=0%). The femoral neck analysis for this type of exercise was heterogeneous (I^2=62%), and the observed effect was non-significant. Trivial and non-significant treatment effects at both lumbar spine and femoral neck were

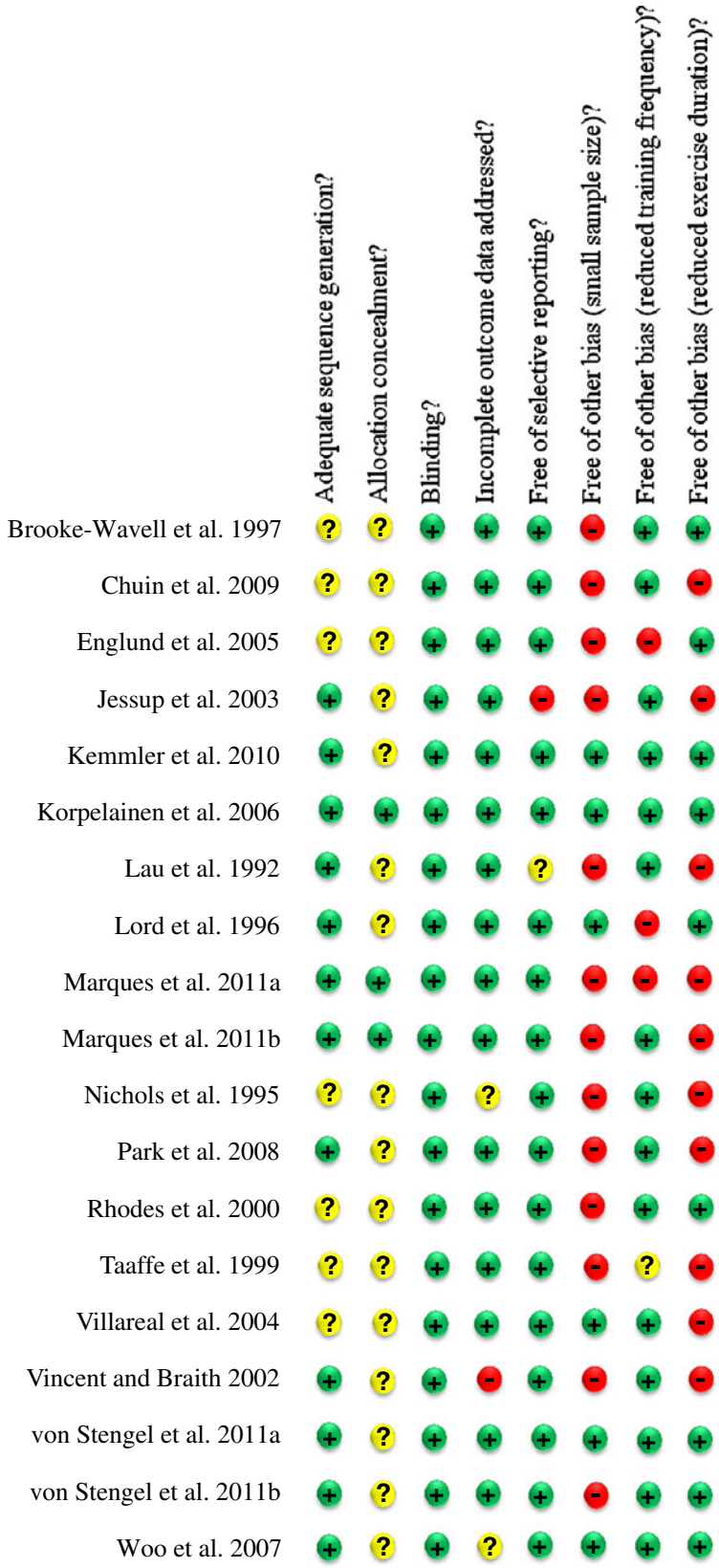

Fig. 2 Risk of bias summary

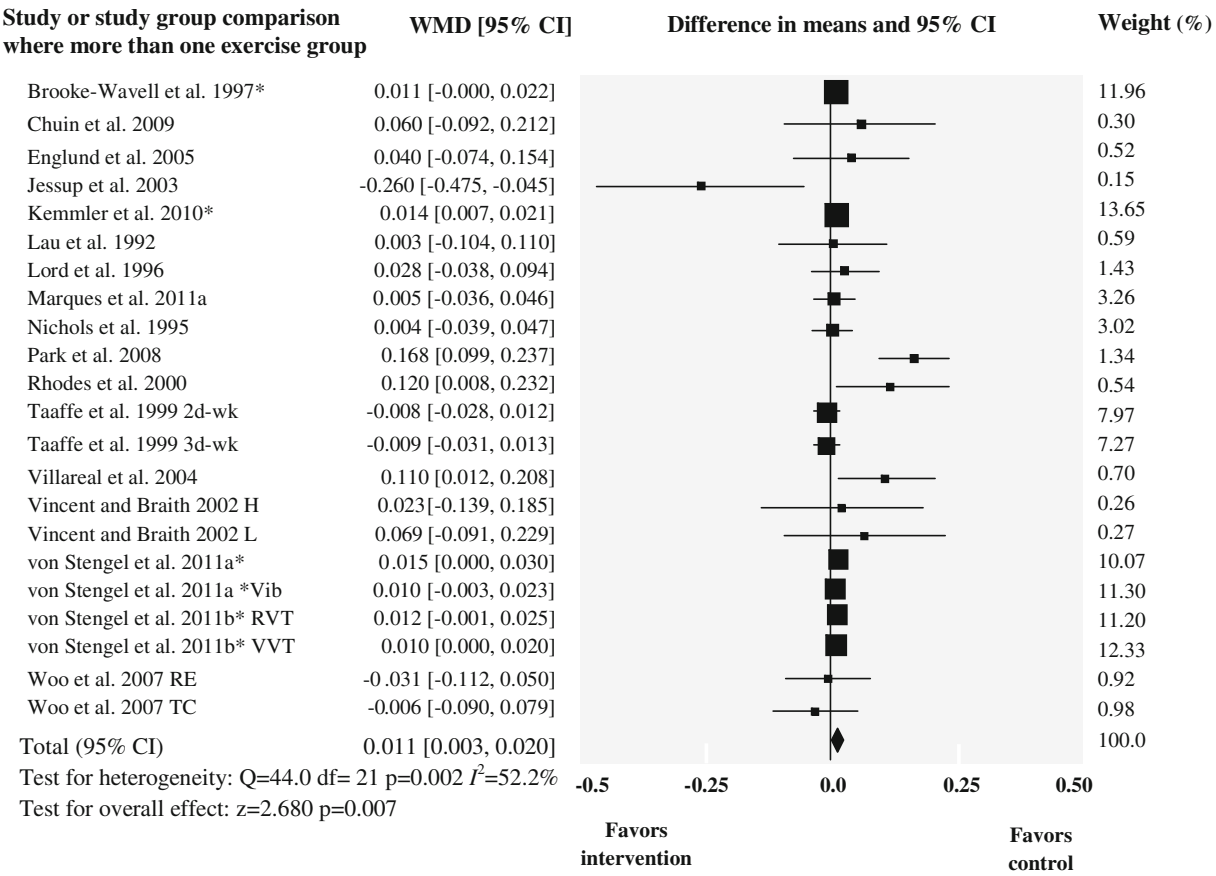

Fig. 3 Forest plot for lumbar spine BMD. *Diamonds* represent overall weighted mean difference (WMD, grams per square centimeter) calculated by random-effect model with 95% CI. *Absolute change values. *RVT* rotational vibration training, *VVT* vertical vibration training, *d-wk* days-week, *H* high, *L* low, *Vib* vibration, *RE* resistance exercise, *TC* Tai Chi

also confirmed (I^2=0%) in the subgroup analyses of protocols evaluating low-impact exercises. Sensitivity analyses for the effects of odd-impact protocols that incorporate group exercise classes were heterogeneous in having a positive effect at the lumbar spine (I^2= 87.5%) and femoral neck (I^2=83.5%). An increase in BMD of 0.039 g/cm^2 (95% CI 0.002 to 0.075; p= 0.038) and 0.036 g/cm^2 (95% CI 0.012 to 0.061; p= 0.004) was observed at lumbar spine and femoral neck, respectively. Finally, the subgroup analyses of trials evaluating protocols of resistance exercise training were consistent in showing no positive and non-significant effects on BMD at lumbar spine (I^2=11.8%) and a positive although also non-significant at femoral neck (I^2=22.3%). Notably, using a random-effects model Q between groups was 3.993 (df=3, p=0.262) in lumbar spine subgroup analysis and Q between groups was 4.322 (df=3, p=0.229) in femoral neck subgroup analysis, which corroborate that treatment effect does not differ among protocols with variations in the skeletal loading characteristics for both bone sites.

Funnel plots for lumbar spine and femoral neck meta-analyses are presented in Figs. 5 and 6, respectively. Visual inspection of these plots seems to indicate no evidence of asymmetry of trials. In the numerical tests, the results of Egger's test showed no evidence of publication bias for lumbar spine (1-tailed p=0.248). Moreover, trim-and-fill method (random-effects model) suggested that two studies were missing to the left side of the mean effect (black circles in Fig. 5); thus the imputed point estimate would reduce the magnitude of the pooled effect size to 0.009 g/cm^2 (difference in means, 95% CI −0.002 to 0.019). Results for femoral neck also showed no evidence of publication bias. Similarly, Egger's test result was not significant (1-tailed p=0.197), while trim-and-fill method suggested that one study was missing to the right side of the mean effect (black circles in Fig. 6), which

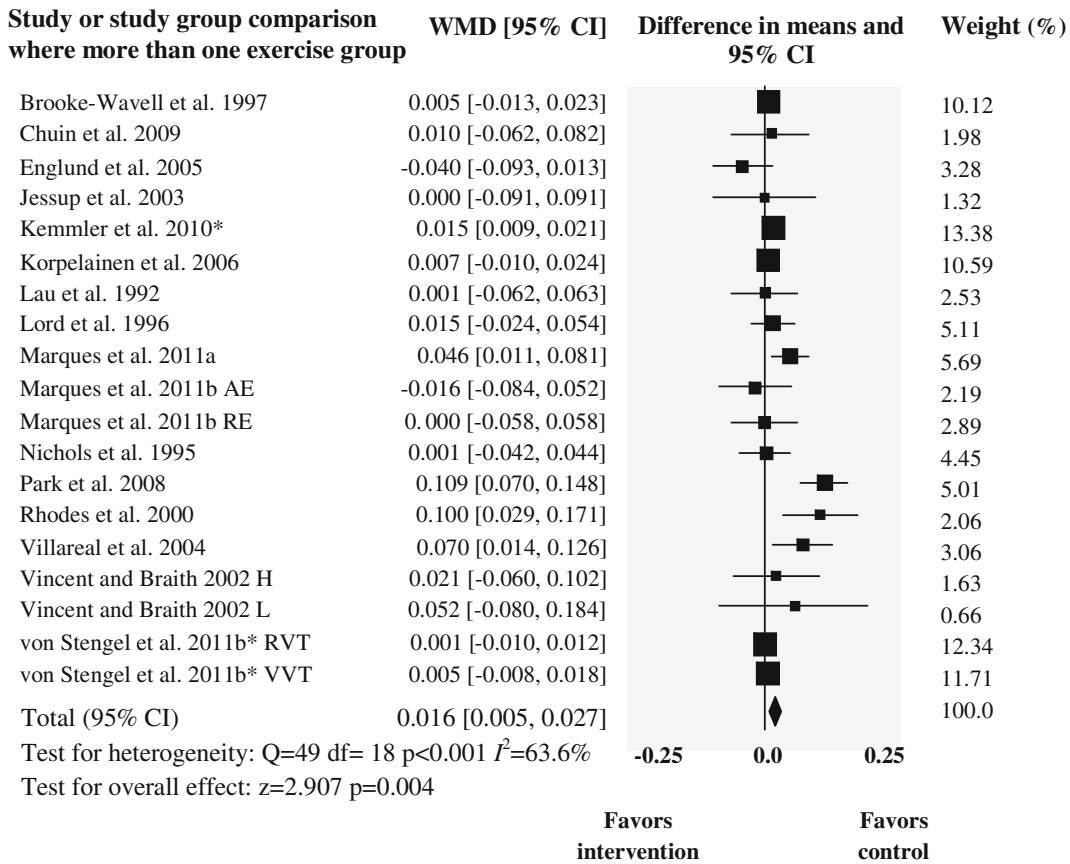

Fig. 4 Forest plot for femoral neck BMD. *Diamonds* represent overall weighted mean difference (WMD, grams per square centimeter) calculated by random-effect model with 95% CI. *Absolute change values. *RE* resistance exercise, *AE* aerobic exercise, *RVT* rotational vibration training, *VVT* vertical vibration training, *H* high, *L* low

increases the magnitude of the pooled point estimate to 0.019 g/cm^2 (difference in means, 95% CI 0.007 to 0.030).

Discussion

Summary of main findings

After many decades of research investigating the association between bone biology, aging and outcomes of exercise interventions, this systematic review and meta-analysis of 19 studies supports the view that exercise of mixed loading impact is associated with significant increases in BMD mean values of 0.011 g/cm^2 (95% CI 0.003 to 0.020 g/cm^2, p=0.007) for the lumbar spine and 0.016 g/cm^2 (95% CI 0.005 to 0.027 g/cm^2, p=0.004) for femoral neck in older adults.

Despite these encouraging results, not all impact exercise protocols appear effective in reducing bone loss (results presented in Table 2). Combined loading studies of impact activity mixed with high-magnitude joint reaction force loading through resistance training were effective at lumbar spine (WMD 0.016 g/cm^2, 95% CI 0.002 to 0.036; p=0.028), and no inconsistency existed among these trials. Odd-impact protocols were also effective in increasing BMD at lumbar spine (WMD 0.039 g/cm^2, 95% CI 0.002 to 0.075; p=0.038) and femoral neck (WMD 0.036 g/cm^2, 95% CI 0.012 to 0.061; p=0.004), and changes were larger, although heterogeneity was evident (I^2=87.5% and I^2=83.5%, respectively). The observed high I^2 may be related with the small sample sizes of two studies (Jessup et al. 2003; Lau et al. 1992) which also limited the statistical power to detect significant differences. Moreover, those studies did not report the attendance rate, which may also affect the post-training results. We found

Table 2 Summary of meta-analyses and sensitivity analyses (random-effects) by region of interest

Analysis	No. study group comparisons	Sample size: EG/CG	Heterogeneity (Q, p-value)	Inconsis-tency (I^2)	WMD, 95% CI	Test of null (Z value, p-value)
All included trials						
Lumbar spine	22	769/571	Q=44.0, p=0.002	I^2=52.2	0.011 g/cm^2, 0.003 to 0.020	Z=2.630, p=0.007
Femoral neck	19	651/541	Q=49.4, p<0.001	I^2=63.6	0.016 g/cm^2, 0.005 to 0.027	Z=2.907, p=0.004
Final means						
Lumbar spine	22	769/571	Q=42.5, p=0.004	I^2=50.6%	0.016 g/cm^2, −0.004 to 0.036	Z=1.593, p=0.111
Femoral neck	19	651/541	Q=43.2, p=0.001	I^2=58.3%	0.020 g/cm^2, 0.003 to 0.037	Z=2.276, p=0.023
Only females						
Lumbar spine	17	575/467	Q=44.3, p<0.001	I^2=63.9%	0.012 g/cm^2, 0.002 to 0.022	Z=2.259, p=0.024
Femoral neck	16	540/478	Q=44.7, p<0.001	I^2=66.5%	0.014 g/cm^2, 0.003 to 0.025	Z=2.485, p=0.013
Sessions ≥3 dwk						
Lumbar spine	18	638/446	Q=39.8, p=0.001	I^2=57.3%	0.013 g/cm^2, 0.004 to 0.022	Z=2.795, p=0.005
Femoral neck	16	532/422	Q=42.1, p<0.001	I^2=64.4%	0.016 g/cm^2, 0.005 to 0.028	Z=2.762, p=0.006
No supplements						
Lumbar spine	14	410/308	Q=32.3, p=0.002	I^2=59.7%	0.018 g/cm^2, −0.003 to 0.039	Z=1.671, p=0.095
Femoral neck	13	385/326	Q=38.2, p<0.001	I^2=68.6%	0.022 g/cm^2, 0.001 to 0.044	Z=2.063, p=0.039
Total sample size ≥50						
Lumbar spine	13	631/463	Q=26.3, p=0.010	I^2=54.3%	0.015/cm^2, 0.006 to 0.025	Z=3.116, p=0.002
Femoral neck	8	443/405	Q=33.0, p<0.001	I^2=78.8%	0.024 g/cm^2, 0.009 to 0.039	Z=3.164, p=0.002
Combined loading						
Lumbar spine	6	362/296	Q=4.5, p=0.482	I^2=0%	0.016 g/cm^2, 0.002 to 0.030	Z=2.203, p=0.028
Femoral neck	4	269/248	Q=7.9, p=0.048	I^2=62.0%	0.014 g/cm^2, −0.011 to 0.040	Z=1.109, p=0.268
Low-impact						
Lumbar spine	5	218/132	Q=1.2, p=0.876	I^2=0%	0.009 g/cm^2, −0.020 to 0.024	Z=1.283, p=0.200
Femoral neck	2	125/85	Q=0.6, p=0.904	I^2=0%	0.002 g/cm^2, −0.048 to 0.045	Z=0.182, p=0.856
Odd-impact						
Lumbar spine	4	75/76	Q=24.0, p<0.001	I^2=87.5%	0.039 g/cm^2, 0.002 to 0.075	Z=2.075, p=0.038
Femoral neck	4	143/141	Q=24.3, p<0.001	I^2=83.5%	0.036 g/cm^2, 0.012 to 0.061	Z=2.883, p=0.004
RE						
Lumbar spine	7	114/67	Q=6.8, p=0.340	I^2=11.8%	−0.002 g/cm^2, −0.019 to 0.015	Z=−0.217, p=0.828
Femoral neck	6	114/67	Q=6.4, p=0.266	I^2=22.3%	0.023 g/cm^2, −0.009 to 0.054	Z=1.414, p=0.157

EG exercise group, *CG* control group, *RE* resistance exercise, *dwk* days/week, *WMD* weight mean difference

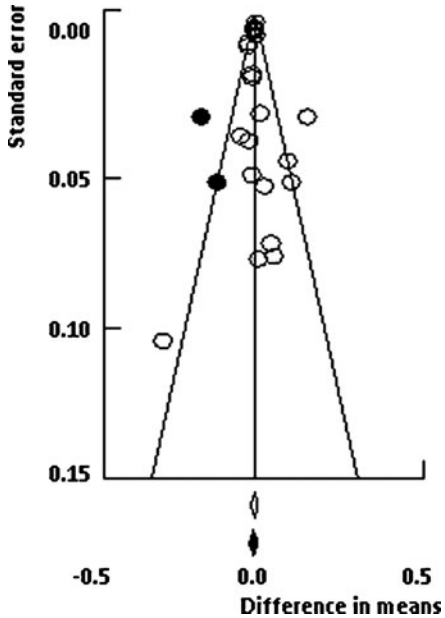

Fig. 5 Funnel plot test exploring publication bias (random-effects model). *Black circles* represent the studies imputed when the trim-and-fill method was applied

consistency among results for low-impact and RE studies on lumbar spine and femoral neck, although non-significant BMD changes were evident amongst these types of protocols at any site and amongst the RCTs that provided a combined loading impact exercise at femoral neck. These findings were less robust and had low power than those for the overall analysis as there

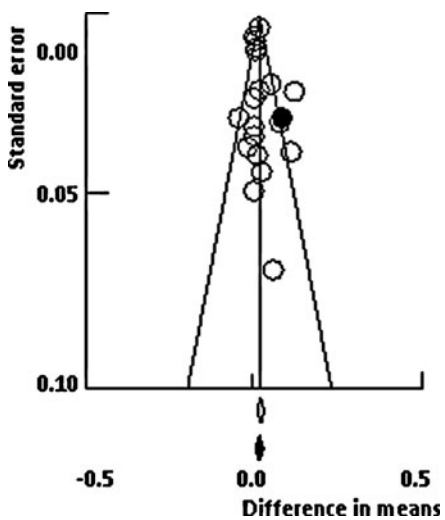

Fig. 6 Funnel plot test exploring publication bias (random-effects model). *Black circle* represents the study imputed when the trim-and-fill method was applied

were fewer studies, which implies that results need to be interpreted carefully. Taken together, subgroup analysis corroborates that treatment effect does not differ among protocols, which lends support to a summary effect (meta-analysis) combining different exercise training with variations in the skeletal loading characteristics for each bone site. However, considering the subgroup analysis our findings indicate that odd-impact loading has the higher potential for preserving BMD at the lumbar spine and femoral neck in older women.

Applicability and quality of the evidence

Our structured systematic searches resulted in 22 RCT study group comparisons evaluating lumbar spine BMD and 19 RCT study group comparisons evaluating femoral neck BMD resulting in an overall sample size of 1340 and 1192 participants, respectively. Though the data from the pooled summaries alone seem to support benefit with exercise protocols, they should be viewed with caution because of the moderate heterogeneity amongst studies for lumbar spine (I^2=52%) and femoral neck (I^2=64%). As systematic reviews bring together studies that are methodologically diverse, heterogeneity in their results is to be expected (Higgins et al. 2002). For example, heterogeneity is likely to arise through diversity in participants' characteristics, doses, lengths of follow-up and study quality. We explored the extent to which heterogeneity affects the conclusions of the meta-analysis through sensitive analyses. Similar magnitude of treatment effects was found in the female-subjects studies, in studies with high training frequency, in studies with no use of supplements and in large-sample size studies. The level of heterogeneity was preserved on all these sensitive analyses. Furthermore, there was considerable variability in the type and dose of exercise prescribed amongst the different intervention trials, all of which may account for the marked variability in the skeletal response to training. Sensitivity analyses were not undertaken on compliance and dropout rates, as values did not diverge extensively.

Other key methodological limitations of the studies, namely aspects of concealment of allocation, should also be considered, as they may limit internal validity. Indeed, only one RCT contained a statement as to whether concealment of allocation had occurred or not, although consolidate standards for reporting of RCTs (CONSORT) are now available to researchers

(Moher et al. 2001), and most of the included trials were undertaken at a time with possible access to these standards. Nevertheless, the lack of reporting these aspects of study quality in our included trials should not be interpreted as that they were not undertaken.

For our meta-analyses, we included RCTs assessing the effect of exercise alone which contributed to the external validity of results, as the additive effects of hormone therapy combined with the exercise may influence the positive findings observed on previous meta-analysis (Martyn-St James and Carroll 2009) and reviews (Lanyon 1996; Kohrt et al. 2004).

The primary outcome for this review was BMD, which is a surrogate marker for fractures (Kanis et al. 2008). The elevated incidence of fractures in older adults is viewed with high concern and may simultaneously act as the driving force for the development and improvement of preventive strategies such as physical activity. However, studies that address the effect of exercise on incidence of fractures are lacking.

Potential biases in the review process

One of the strengths of this review is the comprehensive search strategy that identified a large number of studies from 10 countries. The 19 included trials generally had sound methods and had a low risk of bias, with the main methodological weaknesses being the small sample size (<100 subjects) in 13 trials and reduced duration of the exercise intervention in 10 trials (sustained for less than 12 months). Variation in clinical end-points definition, monitoring and reporting can also be important sources of error. Thus, all included studies measured BMD before and immediately after the exercise intervention, and all assessed the outcome with DXA technology, precluding the effect of variability in inter-instrument reliability. Despite this homogeny, pencil and fan beam technology was used. Although a wide variety of technologies are available for the assessment of osteoporosis, there is a general view that DXA is the "gold standard" and validly measures a real BMD along with other attracting advantages such as high speed, precision, low radiation exposure and availability of reference data (Watts 2004).

Although the methods in most of the trials failed to blind participants, personnel and outcome assessors, this should not represent a limitation, resulting in biased estimates of treatment effect, because of the objective nature of the outcome measurement. Indeed, the magnitude of bias associated with inadequate blinding of participants is likely to be greater for more subjective outcomes (Wood et al. 2008; Higgins and Green 2009).

Because the validity of any meta-analytical review can potentially be compromised by heterogeneity in patient characteristics, we predefined inclusion and exclusion criteria that ensured reasonable likeness between subjects (such as age, health and functional status, diseases and use of pharmacological or nutritional therapy affecting bone).

A random-effects model was used based on our understanding that studies do not share a common effect size (the true effect size varies from one study to the next), and not on the outcome of the test of homogeneity as previously observed (Kelley et al. 2001). This model enables to generalize to a range of scenarios, which is in fact the goal of a meta-analysis. Moreover, to illustrate our findings we used mean differences that are clinically relevant and easy to interpret, as the pooled estimate is expressed in the same unit of the measure technique (grams per square centimeter).

Reported compliance with the exercise interventions was high amongst the trials included in the present meta-analysis. No adverse effects associated with the exercise interventions were reported in any trial, and there were a low number of unsupervised exercise trials.

Regarding publication bias, examination of funnel plots revealed symmetry of study effect sizes for both lumbar spine and femoral neck BMD.

Nevertheless, few limitations should be emphasized. Evidence from intervention trials indicates that BMD and geometry adaptations to loading vary by age, skeletal site and sex. However, due to scant data in older men, we could not synthesize the exercise effects for this population. Moreover, most patients studied were women and Caucasian (although men and other ethnic groups were included), and extrapolation to elderly males and other populations should be made with caution.

Agreements and disagreements with other studies or reviews

The association between exercise and bone health has been widely debated (Kohrt et al. 2004), but the parallel influence of the aging process and related constraints

impose other demanding challenges, namely defining an exercise program well tolerated by older adults and that efficiently stimulate bone remodeling.

To our knowledge, the present study is the first systematic review to include a comprehensive analysis of RCTs assessing the effect of exercise interventions with different impact load characteristics on the lumbar spine and femoral neck sites amongst older adults. An earlier meta-analysis of Martyn-St James and Carroll (2009) also observed that structured exercise protocols of combined loading and exercise programs of low-impact have the potential for preserving BMD at the lumbar spine and femoral neck in postmenopausal women. Moreover, the overall relative change in lumbar spine BMD estimated amongst the included RCTs was small (0.011 g/cm^2) but consistent with other exercise reviews in postmenopausal women (Wallace and Cumming 2000; Martyn-St James and Carroll 2006, 2009). Our findings were also consistent with other reviews also reporting a significant effect of exercise on femoral neck BMD (Martyn-St James and Carroll 2008; Wallace and Cumming 2000; Martyn-St James and Carroll 2006, 2009). However, our results were slightly larger, which is surprising given the absence of high-impact studies, the higher age of participants and the fact that only RCTs were included. It has been recognized that trials employing random allocation methods prevent selection and confounding biases (Akobeng 2005) but will yield more conservative results compared with non-random allocation methods (Moher et al. 2001). When previous reviews restricted the overall analyses to only RCTs study groups, results became non-significant (Martyn-St James and Carroll 2009) or modest compared with controlled trials (Wolff et al. 1999).

Conclusions

Implications for practice

The purpose of this systematic review and meta-analysis was to report more information on exercise training by looking at the body of evidence, for the goal of prescribing optimal exercise regimes as therapy for aging bone loss. The current ACSM position statement on exercise and physical activity for older adults (Chodzko-Zajko et al. 2009) concludes that aerobic exercise training may be effective in counteracting age-related declines in BMD and that high-intensity resistance exercise training preserves or improves BMD. However, those conclusions were based mostly on previous meta-analyses of exercise effects in premenopausal and postmenopausal women (mean age of the postmenopausal subject groups is close to 55–60 years) due to lack of previous meta-analysis in older adults (age >60 years). Furthermore, although the majority of evidence indicates that exercises generating high-intensity loading forces are more effective in increasing BMD (Nikander et al. 2005), none of the RCTs in our review included high-impact loading protocols such as vertical jumps, rope jumping or running at >9 km/h. As older adults compared to young samples (such as premenopausal and postmenopausal women) are more physically prone to injuries due to physiological and functional decline (Tanaka and Seals 2003), high-impact loading protocols incorporating the evidence based in animal models, which supports the notion that greater strain magnitudes provide the most effective stimuli for bone formation (Bailey and Brooke-Wavell 2008), may not easily and effectively be incorporated in exercise prescriptions.

From a clinical point of view, a decrease of 1 SD in femoral neck BMD was associated with an increase in risk ratio for hip fracture by 2.94 in older men and by 2.88 in older women at the age of 65 years (Johnell et al. 2005). Therefore, it seems probable that the increase in BMD reported here might represent a relevant decrease in relative risk for hip fracture.

The largest effect sizes at both lumbar spine and femoral neck were observed in protocols that combined loading studies of impact activity mixed with high-magnitude joint reaction force loading through resistance training (Englund et al. 2005; Kemmler et al. 2010; Lord et al. 1996; Villareal et al. 2004). Accordingly, this specific exercise type may in fact provide a loading stimulus that is both adequate in its strain magnitude and rates and unusual in its loading pattern distributions at these sites. However, because of the differing combinations of skeletal loading activities evaluated in the trials included in these analyses (high inconsistency between trials) and the reduced number of included studies, the recommendation of this type of impact components should be interpreted with caution. Current recommendations regarding optimum exercise for preserving bone mineral density in older adults should advise that

low-impact activities seem to be ineffective in increasing BMD, apart from other physiological and psychological benefits that may be derived from participation in this type of exercise activities.

Implications for research

Future studies of the prevention of bone loss or fracture should not consider low-intensity impact training but, rather, should focus on the optimal combination of impact loading activities (mainly odd-impact loading exercises) and possibly on the improvement of muscle strength and balance impairment by using additional exercises focused on those functional components. Muscle strength and balance outcomes should be reported in addition to bone health end-points. Studies addressing the association between the increments on BMD with exercise loading and the relative risk reduction of fractures risk are required. Studies may also focus on the interaction between exercise and other risk factors for hip fracture (e.g. weight loss, body composition and pharmacological treatments). There is obviously a need for RCTs rather than other types of study, for better descriptions of studies, including the exercise protocol description, specifically the skeletal loading characteristics of the protocols, and trials should consistently apply the standards that are available for reporting of RCTs (CONSORT). Concern about bone health became especially relevant with postmenopausal bone loss; however, given the prevalence of low BMD in men ≥50 years of age (Kiebzak et al. 2002) and the scarce data on exercise effects, it may be especially important to focus on this population. Moreover, there is a lack of studies targeting osteoporotic subjects (irrespective to age), which are required in order to establish the therapeutic effect of exercise in those fragile individuals.

Fifty-eight percent of studies in this review failed to cite ethnicity, and because it is considered a critical variable, this should be reported in future exercise studies. In addition, future research needs to assess and report data on calcium and vitamin D intake because reduced supplies of calcium are associated with a reduced bone mass and osteoporosis, whereas vitamin D deficiency (commonly found in the elderly) leads to a decreased mineralization of bone (Gennari 2001), and there is subsequent confounding potential in relation to exercise-induced changes in BMD.

Considering that the time taken for completion of the bone remodeling cycle (bone resorption, formation and mineralization) is around 3–4 months (Frost 1986) and the varying precision of imaging techniques to assess bone changes in different time intervals, exercise interventions with longer duration (more than 1 year) are clearly advised.

Strength and stiffness are biomechanical parameters typically used to characterize the integrity of bone, which depends on a number of interrelated factors, including bone density, size and shape (Griffith and Genant 2008). Although advances in noninvasive bone imaging techniques, such as peripheral quantitative computed tomography (pQCT) and magnetic resonance imaging (MRI), have been made, the potential of exercise for improving bone strength remains controversial. Thus, there is a need for further well-designed (long-term and adequate sample size) RCTs that properly address this topic.

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[3] Overall Discussion

Overall discussion

The main findings of the studies presented in this dissertation support the hypothesis that exercise training can favorably change BMD, bone turnover, and distinct functional fitness skills related to fall risk in older adults. Our results demonstrated that different types of mechanical loading are effective in reducing the age-related decline on bone mass but also suggested that aerobic exercise training have limited effect on bone density. Moreover, our cross-sectional study lend support to the hypothesis of a fat-bone connection, and more importantly suggesting that appendicular fat mass (AFM) is more relevant than android fat mass to bone health. Paper I failed to demonstrate a clear influence of other previously described risk factors, including lactase nonpersistence, several nutrients, exercise, and knee muscle strength. In accordance with these findings, the secondary outcomes (nutrition, habitual physical activity, body composition, muscle strength) revealed no association with the BMD changes observed after exercise training on papers II, III, and IV. Moreover, paper IV failed to support the positive effect of long-term exercise on bone turnover markers, but a significant role for exercise in inflammatory response was demonstrated. Results did not suggest a association between the change in inflammatory markers and BMD changes after exercise. Our dissertation also covers a systematic review and meta-analysis, which attempted to gather all empirical evidence (that properly fitted the pre-specified eligibility criteria) to answer the research question: is exercise training effective to decrease bone loss or even increase BMD at lumbar spine and femoral neck sites in older adults? Thus, combining information from all relevant studies (including results from papers II and III), the findings from our meta-analysis of RCTs support the efficacy of exercise for increasing lumbar spine and femoral neck BMD in older adults.

Taken together, our studies contributed to the ongoing process of displaying, based on human experiments, evidence on the exercise-bone relationship in the aging skeleton.

Several explanation and mechanisms of action may be linked to the results observed in this dissertation:

Bone-body composition association through fat distribution

The relative contribution of fat mass (FM) and lean mass (LM) to the variation in BMD has been an area of active research, despite the lack of a clear consensus. Moreover, the complexity of these associations rises due to opposite findings regarding the direction of influence between FM and bone, as evidence exist documenting that FM may have a beneficial effect on bone and others suggest that FM may not protect against osteoporosis (Fu et al., 2011; Taaffe et al., 2000; Zhao et al., 2007). The inconsistency of findings may relate to differences in experimental design, sample structure, the use of diverse methods for measuring bone density and, most importantly, to differences in the statistical analysis. Most analyses are confounded by the co-linearity between the studied variables, and therefore have produced misleading results (Reid, 2010). On paper I, our main finding was that AFM, among all included body composition parameters (FM, percent FM, android fat, gynoid fat, android to gynoid fat ratio, appendicular lean mass, and LM) was the most significant predictor of BMD in older women, explaining 23.4% of the femoral neck BMD variance. This was in accordance with the prevailing hypothesis that fat mass accumulation would be more closely related to BMD than LM. A number of mechanistic explanations have been proposed to support the observed epidemiologic and physiologic associations between fat and bone (Reid, 2010; Zhao et al., 2008). The common precursor stem cell that leads to the differentiation of both adipocytes (the cell for storing energy) and osteoblasts, as well the secretion of adipocyte-derived hormones that affect bone development, may partially explain these associations (Zhao et al., 2008). In addition, adiponectin, insulin/amylin/preptin, leptin and adipocytic estrogens are all likely to be involved in this connection (Reid, 2010). The influence of a specific phenotype is still unclear, although it is suggested that android fat, apart from increasing the risk of chronic diseases (such as cardiovascular disease and type 2 diabetes), is deleterious to bone (Blaauw et al., 1996; Jankowska et al., 2001). Our data complies with previous evidence suggesting that subcutaneous fat (i.e. AFM) is more relevant than android fat mass (visceral fat) to bone health. These findings may be relevant to future nutritional and exercise-based approaches to counteract the age-related changes on body composition, mainly low body mass and excessive visceral fat.

In addition, obesity is also associated with chronic inflammation (Frohlich et al., 2000; Visser et al., 1999) being commonly linked to aging as well. Interestingly, it is assumed that factors involved in inflammation are linked with those critical for bone physiology

and remodeling (Arron & Choi, 2000; Lorenzo, 2000). Thus, we were interested in examining the relationship between changes in BMD and inflammatory response after exercise training in older adults. Evidence suggests a long-term anti-inflammatory effect of exercise, which is partly mediated by myokines (mostly the muscle-derived IL-6) and also via a reduction in visceral fat mass (Petersen & Pedersen, 2005). We found that 8 months of a combined exercise protocol (paper IV) elicited a significant decrease in IL-6, hs-CRP and IFN-γ, without a significant reduction in fat mass. Results also supported, at leat in part, the cytokine-mediated effect on bone, as the change in TNF-α was negatively associated with the change in lumbar spine BMD. Taken together, results seem to support the independent effects of exercise training on BMD and inflammatory response without weight loss.

Mechanical loading characteristics influence bone remodeling and subsequently BMD

The most effective way to strengthen bone is by adding new bone tissue where bone stresses are greatest. This occurs when bone adapts to mechanical loading. Bone cells act as mechanotransducers that detect high stresses and signal locally for an anabolic response. However, the main manifestation of aging is the diminution of toughening mechanisms within bone (Peterlik et al., 2006). With aging, the ability of mechanical loads to generate new bone formation declines and this might be due to a loss in the functional capacity of the osteocyte network (Turner, Takano et al., 1995). It is well known that the number of osteocytes within bone tissue decreases with age (Manolagas & Parfitt, 2010). Nevertheless this topic is outside the scope of our dissertation, as we do not attempt to provide new insights into cellular responses (*in vitro* experiments), although they play a crucial role in bone mechanotransduction. On the other hand, we measured BTMs (on paper IV) and the cytokines OPG and RANKL (papers III and IV) to evaluate the rate of bone remodeling, and thus complementing the BMD data (results will be further discussed in the following section).

In line with our research, some modes of exercise and their osteogenic potential have been previously studied, mostly in youth. In fact, the investigation of exercise prescriptions for optimizing peak bone mass have a different rational, although some interchangeable information can be used. Indeed, evidence from different age groups

has demonstrated that regular exercise has effects on bone density, size, and shape, resulting in substantial improvements in mechanical strength (Haapasalo et al., 2000; Milliken et al., 2003; Warner et al., 2006). The positive association between exercise and bone mass has prompted many physicians and public health officials to recommend that individuals should engage in daily exercise, with the goal of reducing the incidence of osteoporotic fracture, and the morbidity/mortality that ensues (Chodzko-Zajko et al., 2009; Kohrt et al., 2004; Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society", 2010; Rosen, 2005). Moreover, a number of studies from both the clinic and laboratory have been gathered, and common threads have emerged that allowed the definition of three rules that govern bone adaptation (regardless to age), which were considered in our experimental work. Briefly, adaptive bone response requires dynamic rather than static mechanical stimulation (Lanyon & Rubin, 1984) (rule 1). High-impact (greater peak strain magnitudes) exercises that produce large rates of deformation of the bone matrix best drive fluid through the lacunar-canalicular network system. In addition, both loading frequency (cycles per second) and strain rate are important determinants of bone adaptation (Turner et al., 1994; Turner, Owan et al., 1995). Each of these components can influence fluid flow vectors within the lacuno-canalicular network. Prolonged exercise has diminishing returns, thus extending the duration of skeletal loading does not yield proportional increases in bone mass (Rubin & Lanyon, 1984) (rule 2). Moreover, bone cells will resensitize to loading if they are given a period of rest between loading bouts. After 24 h of rest, 98% of bone mechanosensitivity seem to return (Robling et al., 2001). Finally, bone cells accommodate to routine loading (rule 3), thus adaptive bone responses require an unusual pattern of bone loading. Each mechanosensitive cell must have some stimulus threshold above which a mechanical signal causes a cellular response (Robling et al., 2006).

Moreover, bone adaptation is a site-specific phenomenon; therefore exercise mode should be designed specifically to target the bone of interest (Kohrt et al., 2004). This concept is supported by numerous human studies examining skeletal health indices in athletes engaged in exercise characterized by sport-specific loading patterns (e.g. tennis), where the long bones of dominant arms display significantly greater BMD and cortical bone content than the non-dominant limb (Ducher et al., 2005).

Considering the above knowledge, and although all four exercise interventions studied in this dissertation were designed in accordance to those rules, different bone

responses were expected to occur as exercise interventions offered different strain rates and magnitudes.

The weight-bearing exercise protocol studied on paper II included marching, step basic movement from a 15-cm bench, and heel-drops with average GRF of 1.7 times body weight (BW), 1.8 times BW, and 2.7 times BW, respectively. Therefore, it may be assumed that those exercises combined with the odd-impact loading, such as step movements, bounding exercises, agility exercises and games with movements including directional elements that the body is not normally accustomed to, had sufficient compressive strain (using GRFs as an estimate) to induce positive and significant skeletal response (femoral neck site).

Although it may be assumed that low-impact loading such as walking produces little effect (i) on bone development (Daly, 2007) and (ii) as a singular exercise therapy for postmenopausal bone loss (Martyn-St James & Carroll, 2008), the American College of Sports Medicine (ACSM) position stand on physical activity and bone health recommends regular weight-bearing endurance activities in conjunction with resistance activities for preserving bone mass in elderly women (Kohrt et al., 2004). However, results of previous meta-analyses reported conflicting results on walking effects on BMD (Bonaiuti et al., 2002; Palombaro, 2005). Of note, the most common and singular exercise activity in older adults is walking (Feskanich et al., 2002). Regarding the evidence of resistance training, results seem to point towards a more consistent and positive direction (Guadalupe-Grau et al., 2009; Martyn-St James & Carroll, 2006). This is in agreement with our findings in a cohort of older women (paper III and IV). It is hypothesized that resistance exercise produce high-magnitude joint reaction force loading, and the intensity (85% 1RM) and selected exercises (which insert on or near the greater trochanter of the femur) effectively stimulates osteogenesis. Finally, our meta-analysis (paper V) also supports the view that not all impact exercise protocols appear effective in reducing bone loss, particularly low-impact activities (results were consistent on showing no significant effects). The largest effect sizes at both lumbar spine and femoral neck were observed in protocols that combined loading studies of impact activity mixed with high-magnitude joint reaction force loading through resistance training (Englund et al., 2005; Kemmler et al., 2010; Lord et al., 1996; Villareal et al., 2004). This is in agreement with our findings in a cohort of both elderly men and women (paper IV). We believe that the incorporation of a combined loading may be not only effective for bone mass renewed but also for improving balance and muscle strength.

It is our belief that exercise strategies designed to take advantage of specific biomechanical principles and specific adaptive responses of cellular networks described in animal and in *in vitro* experiments will ultimately provide novel strategies to strengthen the skeleton to resist fracture.

Evidence on exercise effects on bone remodeling

As previously mentioned, mechanical loading has profound influences on bone remodeling. Investigation of the association between exercise and BTMs has largely focus on comparisons between trained subjects and untrained controls. There are numerous experiments on bone metabolism markers after acute exercise, but not after long-term training (Banfi et al., 2010). Although limited studies have been performed to determine the physiological mechanisms behind bone formation/resorption after long-term training in older adults through an evaluation of bone turnover (Bemben et al., 2010; Vincent & Braith, 2002b), some possible pathways are suggested to explain the metabolic changes observed within the bone. A variety of types and magnitudes of mechanical stimuli have been shown to influence human bone cell metabolism *in vitro*, including fluid shear, tensile and compressive strain, altered gravity and vibration (Scott et al., 2008). A four-stage cell-mediated theory of mechanotransduction is widely accepted, which includes: (1) mechanocoupling - the conversion of physiological loads applied to tissues into local mechanical signals experienced by bone cells; (2) biochemical coupling - the process whereby cells sense a load using mechanoresponsive structures and transform it into a biochemical response; (3) signal transmission - the resultant downstream signaling within and between cells; and (4) effector response of osteoblasts and osteoclasts - the cellular outcomes that lead to build-up, remodeling or resorption of bone matrix (Scott et al., 2008). Our experiments focus on the last stage, as remodeling process comprises activation–resorption–formation phases in which osteoclasts produce bone degradation products that are also released into the circulation and are eventually cleared via kidney metabolism, and osteoblasts synthesize a number of cytokines, peptides and growth factors that are released into the circulation (Brown & Josse, 2002). Their concentration thus reflects the rate of bone turnover.

Several *in vitro* experiments involving the direct application or manipulation of mechanical stimulus to human bone cells were essentially consistent in demonstrating

that mechanical strain, vibration or fluid flow can induce an adaptive response in osteoblasts represented by (high) proliferation, and secretion and mineralization of the extracellular matrix (Burr et al., 2002; Hill et al., 1997; Turner et al., 1994; Turner, Owan et al., 1995). Thus, the major components of the organic matrix (type I collagen, osteopontin and OC) and its associated minerals (hydroxyapatite) may be expected to change in response to loading. Indeed, collagen I, OC and osteopontin secretion (bone formation markers) were generally higher in strained as opposed to unstrained cultures (Scott et al., 2008). Our findings showed that OC (a 5.8 kDa hydroxyapatite-binding protein exclusively synthesized by osteoblasts, odontoblasts and hypertrophic chondrocytes) and beta-CTX (also known as CrossLaps) did not change significantly with training, suggesting no significant changes in bone metabolism. However, it may also reflect the low sensitive of these markers to the specific type, frequency and duration of training. In fact, the exercise-stimulus induced significant changes in BMD, thus, alterations on BTMs are obviously implied in this remodeling process. It is possible that the results of intermediate assessments may have been more sensitive to training than only pre and post-training (8-month) testing. Moreover, the OC to CTX ratio improved 5%, which suggest a trend for increased osteoblastic activity.

The molecular mechanisms for the regulation of osteoclastic and osteoblastic activity involve, as summarized on chapter 1, three main players: RANKL, receptor activator of NF-κB (RANK), and OPG, which are members of the TNF and TNF receptor super family. There is evidence to suggest that OPG and RANKL may be important regulators of load-induced bone formation/resorption by coupling osteoblastic and osteoclastic mechanisms. Briefly, mechanical stimulation may inhibit osteoclast formation and activity by changing the OPG/RANKL ratio in favour of OPG (Rubin et al., 2003; Saunders et al., 2006). In paper III and IV we found no significant interaction or main effects of group and time on serum OPG and RANKL levels or the OPG/RANKL ratio after eight months of either resistance exercise, aerobic exercise or combined exercise (resistance plus multicomponent weight-bearing impact training). Results support some previous studies (Esen et al., 2009) that also failed to corroborate the positive results from *in vitro* experiments testing the effect of mechanical stimulation on RANKL (inducing down-regulation) and OPG expression (showing that various types of stress and strain increases OPG levels (Kim et al., 2006; Kusumi et al., 2005; Saunders et al., 2006).

Fall prevention through muscle strength and balance improvements

In the case of fall prevention it appears that a number of causes exist related to exercise-induced benefits on muscle strength and balance by which fracture prevention might occur. Muscle force appears to be an essential component of the bone-loading milieu. The effect of muscle strength on the bone density is considered to be site-specific since the muscle directly affects the bone to which it is attached (Blain et al., 2001; Iki et al., 2002). Moreover, the relationship of age-associated reductions in muscular strength to skeletal disability has also been suggested (Blain et al., 2001). According to mechanostat theory, declining muscle strength in aging individuals decreases loads on bone that has previously adapted to strains generated by stronger, young adult muscle contractions. The resultant reduction in muscle-imposed bone strain to level below the "minimum effective strain" (MES) constitutes a form of bone unloading so that disuse-osteopenia ensues (Frost, 2003). According to this reasoning, as long as muscle strength continues to decrease, bone will lose mass. Something of a conundrum remains, however, as muscle strength gains following resistance training programs are not always accompanied by gains in BMD (Bemben et al., 2000; Pruitt et al., 1995). Results from our paper II and III suggested that quadriceps muscle strength significantly increased after RE and weight-bearing multicomponent exercise program concurrent with increased femur BMD. However, changes in muscle strength were not significantly related with BMD changes. It seems most likely that declining muscular function acts as one of several important contributors to age-related bone loss of mechanical stimulation.

It is important to recognize that exercise can effectively reduce fracture risk even without dramatic effects on bone mass, thus, the key for reducing many osteoporotic fractures is protecting the skeleton from trauma by preventing a dangerous fall or at least reducing the frequency of falls (Sherrington et al., 2008). Several studies in older adults have demonstrated that proper exercise can reduce falls by improving balance, postural stability and muscle strength (Ballard et al., 2004; Barnett et al., 2003; Carter et al., 2001; Carter et al., 2002; Day et al., 2002; Mochizuki et al., 2006). Indeed, as previously described on chapter 1, impaired mobility and gait, which includes muscle weakness (muscle strength, endurance, and power), gait deficit, balance deficit, and difficulty in rising from a chair are key risk factors for falls amongst older adults (see table 5 pp. 50), with a close relationship to exercise. The underlying mechanisms which explain exercise-induced balance improvements are related, at least in part, with the

sensory systems (somatosensory, visual and vestibular) involved on postural control (Sturnieks et al., 2008). In addition, these systems are known to be affected by aging (Du Pasquier et al., 2003), with diminished muscle strength, decreased muscle volume and mass, loss of muscle fibers, alterations in the motor units (Porter et al., 1995), changes in posture (Sullivan et al., 2009) and decreased balance control (Shaffer & Harrison, 2007).

Although it is suggested that general exercise programs are less effective than programs that target a specific system (e.g. visual, vestibular, somatosensory) closely related to balance maintenance (Howe et al., 2007), our findings demonstrated that even exercise protocols that not included a specific balance component were effective in increasing balance (paper III). Our results demonstrated that both resistance and aerobic exercise resulted in increased static and dynamic balance (including force platform–based measures such as decreased EA and slower velocity of COP displacement). Nevertheless, the effect of both exercise protocols of papers II and IV, that specifically included balance training, targeting mechanoreceptors for limb joint proprioception stimulation were, in agreement with the compelling evidence, effective on improving balance. Moreover, the musculoskeletal system is also deeply involved in balance control (Shaffer & Harrison, 2007). However, one of the most striking effects of age is the involuntary loss of muscle mass, strength, and function (Roubenoff & Castaneda, 2001). Muscle force levels under isometric, concentric, and eccentric conditions decline from age ≈40 yr and accelerate after age 65–70 yr (Chodzko-Zajko et al., 2009). Diminished muscle function with age is a consequence of loss of muscle mass and decreased power. Loss of muscle fiber number is the principal cause of sarcopenia, although fiber atrophy, particularly of type II fibers (Lexell, 1995), is also involved. Sarcopenia is related to denervation, leading to loss of motor units, decrease production of anabolic hormones (such as testosterone, growth hormone and IGF-1) (Goldspink & Harridge, 2004), and increase in the release of catabolic agents, specifically interleukin-6, amplifying the rate of muscle wasting (Roubenoff, 2003). Deficits in strength and power predict disability in old age and mortality risk (Janssen et al., 2002; Reid et al., 2008). Similarly, there is evidence to suggest that chronic exercise might induce positive changes in muscle strength in older adults, with reported increases ranging from less than 25% (Carmeli et al., 2000; Hakkinen et al., 2001) to greater than 100% (Ferketich et al., 1998; Lexell et al., 1995). This fact has been attributed, to a greater extent, to neural adaptations especially observed during the earlier weeks of training, and to hypertrophy of muscle fibers developed during the

overall months of training (this for mostly resistance exercise) (Macaluso & De Vito, 2004). This is in agreement with our findings (paper III), as only the resistance exercise group significantly increased their maximal KE and KF torques at both angular joint speeds, whereas no significant alterations occurred after aerobic exercise training. Moreover, women involved in the multicomponent exercise program studied on paper II showed a clear trend to improve (mean change 2.24-9.09 Nm) in all maximal strength tests for both limbs, but only increased significantly their maximal KF torque at 180°/S (left limb).

Methodological discussion

Some methodological decisions warrant a special comment due to some underlying limitations or in other cases due to their pertinent contribution to the novelty of this dissertation.

It is well known that besides BMD, mechanical stress also improves bone strength by influencing collagen alignment as new bone is being formed (Burr et al., 2002; Robling et al., 2002). There are a number of biomechanical parameters that can be used to characterize the integrity of bone: stiffness, ultimate force (Fu), work to failure (U), and ultimate displacement (du) (Turner, 2006), but their assessment is limited to laboratory studies. Bone mineral content (BMC) and BMD are related to bone strength, but sometimes inferring strength from bone-mineral measurements can be misleading. Instead, assessment should include some measure of bone shape and size. As briefly described on chapter 1 (Theoretical background) a number of methods are currently available to assess bone mass and architecture. The ideal scenario would have been using CT because it analyzes both densitometry and geometrical components either of the entire bone or its cortical and trabecular components separately, and estimates bone strength by FEA. However, compared to DXA, CT would imply a very high cost, which made this option inaccessible.

An important contribute to improve the accuracy of the measurements of the studies comprised in this dissertation was the use of an objective measure of physical activity by accelerometers. These motion sensors, which records both the number and magnitude of vertical accelerations generated by human movement have been validated in older people (Harris et al., 2009). Self-report measures do not acquire activity patterns throughout the day, and perceptions of the intensity of any stimulus

depend on the individual experience. In older adults in particular, self-report may also be influenced by fluctuations in health status and mood, depression, or anxiety ability (Rikli, 2000) and by problems with memory and cognition (Harada et al., 2001; Stewart et al., 2001). Objective methods such as accelerometers provide considerably greater precision of measurement, as they overcome older adults' lack of ability to sometimes recall and quantify physical activity. However, physical activity is difficult to measure because it is a complex behavior, particularly on bone research field (not all physical activities influence bone in the same way or to the same extent). Thus, the lack of significant association between physical activity and femoral neck BMD (finding from paper I) may have been attributed, as previously discussed, to the fact that some potentially osteogenic-exercise stimulus such as weight-bearing exercise may not be appropriately captured by accelerometers (strain magnitude and distribution).

Another important methodological option was the use of force platforms to accurately detect relevant biomechanical changes in postural stability (Chaudhry et al., 2011), which has been scarcely used to assess postural balance in older people.

Moreover, lactase nonpersistence (genetically defined by the C/T -13910 genotype), which has been associated with bone density (Obermayer-Pietsch et al., 2004) and may represent a genetic risk factor for bone fractures for older adults (Enattah et al., 2005), was therefore included as a potential confounding variable. However, few previous prospective studies have considered this factor in the analysis of the primary end-points.

An isokinetic dynamometer, which provides constant velocity with accommodating resistance throughout a joint's range of motion, was used to measure the strength produced by the muscles, namely knee extensors and flexors, under dynamic conditions. It has acceptable mechanical reliability and validity (Drouin et al., 2004).

Pharmacological interventions are important contributors to the bone loss observed in osteoporosis (Pitts & Kearns, 2011), and are more common among older adults, as they represent a high proportion of patients suffering from chronic debilitating diseases for which drug intervention is necessary. Following this rationale, our selection criteria excluded subjects taking any drug known to be associated with osteoporosis such as, glucocorticoids, selective serotonin reuptake inhibitors, anticonvulsants, and proton pump inhibitors (Pitts & Kearns, 2011). For example, glucocorticoids are used in the treatment of inflammatory and autoimmune diseases, neoplasias, and following organ transplantation, and are the drugs causing osteoporotic fractures most frequently (Mazziotti et al., 2010). The stimulatory effect of glucocorticoids on bone resorption *in*

vivo may involve the induction of IL-6 receptors in skeleton cells (Dovio et al., 2006), which could lead to more pronounced IL-6 effects (increase osteoclast recruitment) in corticoid-treated subjects. Glucocorticoids at high concentration inhibit IGF-I synthesis in osteoblasts by a transcriptional mechanism (Canalis, 2005). Yet, there is little definitive information on whether aging makes bones more vulnerable to pharmacological agents that have been shown to cause bone loss and susceptibility to fracture. However, various changes in physiological processes and in bone itself with aging would lead to the prediction that there would be alterations in the response of these drugs in the elderly individuals. On the other hand, several therapeutic drug-based options are available, such as bisphosphonates, the SERM raloxifene, PTH, estrogens, and calcitonin (Brewer et al., 2011). In general, all these bone remodeling agents are associated with increased BMD and a reduction in rate of fractures (Brewer et al., 2011). Thus, we predefined the exclusion of participants using any drug therapy for bone loss/osteoporosis, since we were interested on studying exercise as a stand-alone stimulus.

Finally, we performed several biochemical assays to detect the dynamics of the metabolic remodeling and to complement the radiological assessment of skeletal BMD. Although we believe that the positive contribution of these BTMs is indubitably relevant, the results have always to be interpreted with caution. The majority of these molecules are present in other tissues besides the bone and their measured levels in serum may be influenced by nonskeletal processes (Civitelli et al., 2009). The serum measurement of RANKL and OPG remains somewhat difficult. The proportion of circulating OPG is monomeric, dimeric, or bound to RANKL are difficult to quantify, and there is some uncertainty to select the most biologically relevant form to measure (Garnero, 2008). The same issues arise for the measurements of circulating RANKL which, in its free form, is barely detectable in healthy individuals (Garnero, 2008).

We ought to point-out that the small number of older adults involved and short duration of studies precluded an assessment of the interventions on falls and fracture. It is possible that the aggregate beneficial effects of exercise training on muscle strength, balance, and BMD could lower the risk of falls and fractures.

The **limitations** of our experimental work were described in detail on all five papers. The **implications for practice and for research** were presented under specific sections on paper V (systematic-review and meta-analysis, see pp. 125).

[4] Conclusions

Conclusions

Based on the results that emerged from the present dissertation we emphasize the following conclusions: **1**) appendicular fat mass seems to be consistently more important than total fat mass or abdominal fat for femoral neck BMD in older women, after adjustment for age, height, age at menopause, potential renal acid load, physical activity and knee muscle strength; **2**) eight months of a weight-bearing exercise program leads to an improvement in strength, balance and femoral neck BMD that may counteract the age-related increased incidence of falls and fractures; **3**) eight months of a RE program leads to an improvement in balance, strength and hip BMD, while the AE program only demonstrate to protect against the functional balance control that is strongly related to fall risk; **4**) eight months of exercise training may have a positive impact on body composition (reduction of fat mass and increase of lean mass). However, AE training seems not grant the same significant effect; **5**) long-term RE and AE training in older adults are insufficient for modifying serum concentrations of OPG and RANKL; **6**) bone turnover markers, OPG and RANKL remained unchanged, whereas inflammatory cytokines (IL-6, hs-CRP and IFN-γ) decreased significantly after 8 months of a combined exercise training program involving RE and weight-bearing exercises; **7**) the systematic review and meta-analysis of 19 studies supports the view that exercise of mixed loading impact is associated with significant increases in BMD mean values of 0.011 g/cm^2 for the lumbar spine and 0.016 g/cm^2 for femoral neck in older adults.

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