Tesis Doctoral Europea / European Doctoral Thesis

VARIACIONES EN EL CONTENIDO MINERAL, DENSIDAD Y GEOMETRÍA DEL FÉMUR DE RATAS POR EFECTO DE LA CANTIDAD Y FUENTE PROTEICA, ENTRENAMIENTO DE FUERZA Y DE LA ADMINISTRACIÓN DE ESTEROIDES ANABOLIZANTES

MINERAL CONTENT, DENSITY AND GEOMETRY IN THE RAT FEMUR AS A RESULT OF THE AMOUNT AND SOURCE OF PROTEIN, RESISTANCE TRAINING AND ANABOLIC STEROIDS ADMINISTRATION

DEPARTAMENTO DE FISIOLOGÍA
FACULTAD DE FARMACIA
UNIVERSIDAD DE GRANADA

ELENA NEBOT VALENZUELA

2013

Editor: Editorial de la Universidad de Granada
Autor: Elena Nebot Valenzuela
D.L.: GR 1900-2013
ISBN: 978-84-9028-612-8

INDEX

RESUMEN

Existe un interés creciente en la población por mantener un adecuado estado de salud y prevenir enfermedades, para lo cual es imprescindible alcanzar un estado nutricional adecuado y combinarlo con actividad física. Estos dos factores deben estar balanceados, pero tanto en uno como en otro se han introducido prácticas sobre las que hay un debate científico con resultados contradictorios, en los que merece la pena profundizar. La mayor controversia se produce en los estudios a nivel de estructura y de metabolismo óseo.

Una práctica habitual en nuestros días es un consumo elevado de proteínas, que pueden ocasionar problemas de salud derivados de las alteraciones que se producen a nivel metabólico, hepático, renal y óseo.

A pesar de las alteraciones producidas por el exceso de proteína, hay diferencias según la fuente proteica empleada en la dieta, sobre todo a nivel óseo. Dietas ácidas como las que aportan una proteína de fuente animal, como la proteína de lactosuero, son dietas que pueden comprometer las propiedades mecánicas del mismo. Las proteínas de origen vegetal (soja) producen dietas más alcalinas que, además, aportan otros nutrientes y compuestos de carácter antioxidante.

La práctica de un ejercicio físico se considera como un medio para mejorar la salud. La función de la actividad física sería la de curar o evitar la aparición de enfermedades, especialmente aquellas que se asocian con el sedentarismo (enfermedades hipocinéticas). Esta concepción se basa en el hecho de que el gasto energético asociado a la actividad física puede provocar determinadas adaptaciones orgánicas, consideradas factores de protección frente a las enfermedades.

Otra práctica habitual en los deportistas es la administración de esteroides anabolizantes, que aumentan la secreción o la actividad de sustancias que promueven el crecimiento y la síntesis protéica, tienen una accion catabólica. Tambien pueden causar múltiples problemas de salud como alteraciones hepáticas o cardiovasculares.

Por la importancia de todo ello, en la metodología de esta Tesis Doctoral, las intervenciones realizadas han sido:

1.- Influencia de la cantidad de proteína de la dieta (normoproteica frente a hiperproteica)

2.- Influencia del tipo de proteína de la dieta (proteína de origen animal- whey protein- frente a proteína de origen vegetal- soy protein-)

3.- Seguimiento de un protocolo de entrenamiento de fuerza (grupo sedentario frente a grupo con ejercicio)

4.- Administración de esteroides anabolizantes (grupo control frente a grupo inyectado diariamente con dosis de 10mg/kg de peso corporal de decanoato de nandrolona)

El objetivo general de esta Tesis Doctoral ha sido analizar los efectos de las dietas hiperproteicas, de la fuente de proteína (lactosuero vs. soja), del entrenamiento de fuerza y de la administración de esteroides anabolizantes sobre las variaciones en el contenido mineral, densidad y geometría del fémur de ratas. Además se han determinado el peso y el contenido de minerales totales y de calcio, fósforo, magnesio y zinc presentes en el fémur y su relación con la utilización digestiva de esos minerales. Se ha comprobado la función reguladora del riñón para preservar la integridad ósea, analizando la excreción urinaria de calcio, de citrato y el pH de la orina. Se han analizado los cambios a nivel del hemograma, niveles plasmáticos de corticosterona y testosterona, así como marcadores óseos de formación y resorción.

Los resultados de la presente memoria de Tesis ponen de manifiesto una serie de conclusiones, entre las que destacan que: a) Una dieta hiperproteica produce cambios en la geometría del fémur, aumentando el área de la metáfisis, así como el área trabecular. En la diáfisis del fémur aumentan el área de la sección transversal, el área cortical y los perímetros del periostio y del endostio. Los cambios a nivel de los perímetros se producen de una manera proporcional, ya que se mantiene el grosor cortical, e indican un aumento de la matriz orgánica del hueso. b) Una dieta hiperproteica contribuye a que el hueso mantenga su contenido en calcio a pesar de la alta excreción renal del catión, acompañándose de una hipocitraturia acusada y de una acidificación de la orina. c) El aumento significativo de los niveles de Mg y Zn en el hueso, con el consumo de dietas hiperproteicas, puede tener una repercusión fisiológica por su función como cofactores de numerosas enzimas. d) El hidrolizado de soja como fuente proteica aumenta la

cantidad de alimento ingerido y, la cantidad de calcio ingerido y absorbido producen cambios relevantes en el hueso frente a la proteína de lactosuero, reflejándose en un mayor contenido de calcio en fémur, y menor contenido del resto de minerales estudiados (P, Mg y Zn), con mayor área cortical y mayor grosor cortical en la diáfisis, lo que sugiere una mayor resistencia mecánica. e) Una dieta a base de proteína de soja mejora la fisiología renal, disminuyendo la excreción urinaria de calcio, aumentando la excreción de citrato y produciendo una orina más alcalina. f) Un entrenamiento de fuerza de alta intensidad produce un retraso en el crecimiento en longitud del hueso. g) Un entrenamiento de fuerza produce un incremento de la BMD de la metáfisis, especialmente a nivel cortical/subcortical, probablemente debido a que las tensiones producidas por el ejercicio en esa zona del fémur posibilitan una disposición de trabéculas que aumenta la densidad, además disminuye el área de la sección transversal, reflejándose este cambio en una menor área trabecular. h) A nivel de la diáfisis, el entrenamiento de fuerza produce disminución en el área y en el grosor cortical que reflejan un remodelado menos dinámico que en la metáfisis y que indica que la cantidad de hueso formado puede no haber sido suficiente para mantener el grosor cortical adaptado apropiadamente al incremento de carga mecánica impuesta por el protocolo de ejercicio. i) Con el entrenamiento de fuerza, aumenta la fosfatasa alcalina, marcador de formación ósea, reflejándose esta mayor actividad osteoblástica. j) La ingesta de alimento y de Ca, P, Mg y Zn es menor por efecto del entrenamiento de fuerza y esta asociada a una menor absorción de estos minerales, debido a los altos niveles de corticosterona circulante, reflejo del fuerte estrés al que están sometidos los animales por este protocolo desarrollado. k) A pesar de los cambios a nivel digestivo y plasmático el entrenamiento ha evitado la movilización de calcio del hueso, el riñón ejerce una función reguladora importante al evitar la pérdida de calcio por orina, sin embargo, el nivel de citrato urinario excretado es menor. l) Se han observado cambios hemáticos como consecuencia del entrenamiento de fuerza, tales como: un aumento del hematocrito asociado a una disminución del hierro sérico, debido a una menor ingesta con menor aporte general de nutrientes, y a los altos niveles de corticosterona que producen un mayor recambio de células sanguíneas. m) La administración de esteroides anabolizantes aumenta los niveles de calcio y magnesio en plasma y disminuyen la excreción renal de calcio, estos cambios favorecen la mineralización ósea, conduciendo a huesos de peso mayor, mayor porcentaje de cenizas y con un contenido mayor de Ca y Mg. n) El efecto de los esteroides anabolizantes no se refleja en un mayor BMD, aunque

sí afecta a la geometría de la metáfisis, con un menor área de la sección transversal, un menor área trabecular y un menor área cortical/subcortical, sin producirse cambios a nivel de diáfisis. o) Aumenta la excreción de citrato urinario, contribuyendo a la alcalinización de la orina, por efecto de la administración de esteroides anabolizantes.

I. INTRODUCTION

1. BONE PHYSIOLOGY

At the gross level, all the bones in the adult skeleton have two basic structural components: compact and spongy bone. The solid, dense bone that is found in the walls of bone shafts and on external bone surfaces is called compact or cortical bone. At joints, compact bone covered by cartilage during life is called subcondral bone.

The second kind of bone has a more spongy, porous, lightweight, honeycomb structure. This bone is found under protuberances were tendons attach: in the vertebral bodies, in the end of long bones, in short bones, and sandwiches within flat bones. This cancellous, or trabecular bone is named after the thin bony spicules (trabeculae) that form it.

Areas of trabecular bone in the growing skeleton constitute sites of the red marrow, a blood-forming or hematopoietic tissue that produces red and white blood cells and platelets. The yellow marrow, mainly a reserve of fat cells found in the medullary cavity of tubular bones, is surrounded by compact bone. During growth the red marrow is progressively replaced by yellow marrow in most of the long bones.

2. BONE MINERAL CONTENT

The important minerals within bone are calcium, phosphorus and magnesium. There is about 1Kg of calcium in the adult skeleton as a complex crystalline material with phosphate in the form hydroxyapatite. This mineral is laid down in an organized manner on an organic matrix, the main constituent of which is collagen. There are also important non-collagen proteins within the skeleton [1].

Of several systematic hormones and local factors affecting bone remodeling, vitamin D and PTH may be the most important factors for regulating bone formation and resorption.

2.1. CALCIUM

The adult skeleton contain about 1kg of calcium and is in equilibrium with the plasma calcium at a concentration about 2.25-2.60 mmol/l. The amount of calcium within the skeleton changes with age, accordins to size and composition, increasing during growth and declining with the bone loss of later years.

Calcium balance and its control

The external balance of calcium is determined by exchange between the skeleton, the intestine and the kidney. These fluxes are controlled by the action of the calciotrophic hormones, parathyroid hormone (PTH), 1,25-dihydroxycholecalciferol, and calcitonin. They are also influenced by a variety of locally acting hormones [1].

The actions of the classic calciotrophic hormones, the locally acting hormones (cytokines) and the hormones controlling hypercalcaemia of malignancy have been extensively reviewed by Marcus et al., 1996 [2].

2.1.1. Vitamin D

All the actions of vitamin D are mediated through its active metabolite $1,25(OH)_2D_3$, except that there is some evidence in animals that the duhydroxilated metabolite $24,25(OH)_2D_3$, is not entirely inert [3]. Although the actions of $1,25(OH)_2D_3$ concern calcium transport and are important in the control of plasma calcium (particularly by controlling the intestinal absorption of calcium and the osteoblastic resorption of bone), it has been shown that the calciotropic function is only one aspect of $1,25(OH)_2D_3$ metabolism. The identification of the specific nuclear cell receptor (VDR) for this metabolite has led to its discovery in a wide range of tissues. Important amongst the non-calcitrophic effects are those on cell differentiation and on the immune system.

Whether vitamin D directly induces osteoblastic bone mineralization is still a matter of controversy, the findings in vitamin D receptor (VDR) knockout mice have shown an important clue for considering this issue [4]. Different results clearly indicate that the primary role of vitamin D is to stimulated intestinal absorption of calcium, which in turn promotes bone mineralization inderictly. It is likely that to vitamin D does no appear to play an important role in the actual mineralization process of the skeleton, and that the failure to mineralize the skeleton in vitamin D deficiency is due to inadequate levels of calcium and phosphorus in the plasma [4].

It appears paradoxical, but vitamin D functions in the process of calcium mobilization from calcified bone, making calcium available to the extracellular fluid upon demand by the calcium homeostatic system. This important observation was first reported by Carlsson, 1952 [5].

Osteoclasts are the only cells responsible fone bone resoption.It is well recognized that osteoclasts are derived from hemopoietic cells of the monocyte-macrophage lineage [6]. Hematopoietic monocytes and macrophages are present in almost all tissues,whereas osteoclasts are present only in bone. The process of osteoclast development consist of

several steps: they are proliferation, differentiation, fusion, and activation of osteoclast. In 1988, Takahashi et al. [7], established that multinucleated osteoclasts were formed only when spleen cells and osteoblastic cells where co-cultured in the presence of $1,25(OH)_2D_3$. The bone elects of $1,25(OH)_2D_3$ depend on its dose levels: physiological doses (0,1 µg/Kg) do not stimulate bone mobilization, this stimulated absorption of calcium but rather inhibed the PTH-induced bone resorption [4], which then stimulates bone mineralization. Supression of the PTH-induced bone resorption by vitamin-D appears to occur by inhibiting the signal transudation pathway of PTH [29]. In order for $1,25(OH)_2D_3$ to induce bone resorption, supraphysiological, or pharmacological doses of the vitamin are required [4].

2.1.2. Parathyroid hormone (PTH) and PTH-related peptide (PTHrP)

PTH controls plasma calcium by its indirect action on intestinal calcium absorption (by controlling 1-α-hydroxylation of 25(OH)D), and its direct effect on renal reabsortion of calcium, and on bone resorption. This last effect is thought to take place via the osteoblast, which contains PTH receptors whilst the osteoclst does not. The direct action of PTH on the cells of the renal tubule and bone is mediated by the classic activation of adenyl cyclase; there is also increasing evidence of action via the phosphokinase C and inositol triphosphate pathway [1].

The secretion and synthesis of PTH is controlled at various stages in its metabolism by plasma calcium concentration, mediated by a very sensitive system of calcium-sensing receptors [8].

There is a hormone whose amino-terminal sequence and biological actions are very similar to those of PTH, the hormone has been labeled PTHrP. There is some evidence that this hormone is important in fetal physiology and early development [1].

PTH stimulates bone formation and resorption [9] and can increase or decrease bone mass, depending on the way of administration [31, 32]. Schneider et al., (2012) [33] observed that continuous infusions and daily subcutaneous injections of parathyroid hormone stimulated bone formation similarly but had different effects on bone resorption and bone mass. Continuous infusions, which resulted in a persistent elevation of the serum PTH concentration, led to greater bone resorption than did daily injections, which caused only transient increases in the serum PTH concentration.

PTH or its amino-terminal fragments and analogues prevent, arrest, or partially reverse bone loss in animals and humans. In animals, PTH induces parallel increases in bone mass

and bone strength, suggesting that treatment with parathyroid hormone may provide protection against fractures in humans [34].

PTH and PTHrP increase bone resorption by stimulating both osteoclastogenesis and activating the mature osteoclast [35-37]. These effects require the participation of classical PTH target cells, including stromal cells and osteoblasts [38]. The mechanisms by which PTH increases bone formation are complex. PTH exert a variety of effects on osteoblasts: increase in osteoblast number because increase expression and release of a number of potent mitogens [10-12], which may act in a paracrine fashion to expand the pool of osteoprogenitors. Also PTH may derive from activation of bone lining cells to become active osteoblasts [13].

2.1.3. Calcitonin

The role of calcitonin (CT) in normal physiology remains a mystery. Measurements of its concentration in the elderly people with or without femoral neck fracture or osteoporosis have been controversial. There is some evidence that it has a role in protecting the skeleton by inhibiting the activity of osteoclasts during times of stress such as growth and pregnancy. It is know that CT and calcitonin gene-related peptide (CGRP) are produced by alternative splicing from a common gene. CGRP is a very active vasopeptide but has negligible effects on calcium metabolism [1].

2.1.4. Local regulators of bone remodeling

2.1.4.1. Locally acting agents: cytoquines

Changes in calcium metabolim are also brought about by locally acting hormones. In addition to the importance of PTHrP in the hypercalcemia of malignant solid tumors, immune cell products such as llymphotoxin, tumor necrosis factor (TNF) and the interleukins promote bone resorption, and a variety of osteotrophic cytokines may have a central role in normal bone remodelling growth. The many factors capable of stimulating bone resorption include tumor necrosis factors α and β, transforming growth factors, epidermal growth factor, platelet-derived growth factor, and arachidonic acid metabolites such acid metabolites such as prostaglandins and leukotrienes [1].

2.1.4.2. Insulin-like Growth Factors (IGFs)

IGFs are ubiquitous in nature and regulate cell growth and death. Outside the liver, bone is the richest source of IGF-I in the mammalian body and play a predominant role in skeletal

modeling and acquisition. Systemic IGF-I, regulated by GH, the IGFBPs, and several protease, as well as the IGF receptors, also must be important for full lineal growth and peak skeletal adquisition. They are many works suggest that recombinant growth factors may be anabolic for the skeletal remodeling unit [14]. First, both GH and IGF-I stimulate osteoblastic differentiation and in vivo models using targeted deletion or over expression of IGFs or the IGF-I support a critical role for this regulatory circuit. In animals models GH and IGF-I stimulate longitudinal growth, bone formation and BMD [14].

2.1.4.3. Prostaglandins

Prostaglandins (PGs) are abundantly expressed in bone, and their production is highly regulated by multiple local and systemic factors that induce COX-2 expression (enzyme related to PGs production) [15]. Studies in cell and organ culture indicate that PGs have both stimulatory and inhibitory effects on bone formation [16]. In vitro, PGE2 generally stimulates the differentiation of both osteoblasts and osteoclasts, but inhibitory effects on bone formation and resorption are also seen. In vivo, the predominant effect of exogenous PG2 are stimulate both bone formation and resorption, but the relative magnitude of these can vary so that in some models there is net bone loss whereas in others there is a gain a bone mass. Some of the complexity of PGE2 actions may be explained by the multiplicity of receptors for PGE2 [17]. The PG in fracture healing and to the development of potential ttherapeutic agents to increase bone mass and accelerate fracture healing. Despite the availability of mice deficient in COX-2, the effects of endogenously produced PGs on bone remodeling under physiologic and pathologic conditions remain elusive. Reasons for this may beredundancy of the PG pathways, as well as potential compensatory mechanisms involving other hormones, such as PTH [15].

2.2. PHOSPHORUS

The distribution, function and control of phosphorus within the body is different from that of calcium and, for those interested in bone, phosphorus often takes a secondary role. Neverthles phosphorus deficiency has widespread effects and there are important bone diseases related to disorders of phosphorus metabolism. An important distinction has been draw between total body deficiency or retention of phosphorus and his redistribution between bone and soft tissue [18].

Phosphorus balance

Phosphorus plays an important role in cellular physiology and skeletal mineralization, serving a constituent of nucleic acids and hydroxyapatite, a source of the high energy phosphate in adenosine triphosphate, an essential element of the phospholipids in cell membranes in cell membranes and a factor influencing a variety of enzymatic reactions [19].

In both organic and inorganic forms is widely distributed throughout the body, nevertheless 85% is contained within the skeleton. Deficiency of phosphate may produce osteomalacia, myopathy, growth failure and defects in leucocyte function [1].

The critical role that phosphorus plays in cell physiology has resulted in development of elaborate mechanism designed to maintain phosphate balance. These adaptatives changes are manifest by a costellation of measurable responses, the magnitude of which is modified by the difference between metabolic Pi need and exogenous Pi supply. Such regulation maintains plasma and extracellular fluid phosphorus within a relative narrow range and depend primarly on gastrointestinal absorption and renal excretion as mechanism to affect homeostasis. Although investigators have recognized a variety of hormones that influence these various processes in concert with associated changes in other metabolic pathways, the sensory systems, the messenger, and the mechanisms underlying discriminant regulation of Pi balance remain incompletely understood [19].

Whereas log-term changes in Pi balance depend on these variables; short-term changes in phosphate concentrations can occur as a result of redistribution of phosphate between the extracellular fluid and either bone or cell constituents. Such redistribution results secondary to various mechanisms, including elevated levels of insulin and/or glucose; increased concentrations of circulating catecholamines; respiratory alkalosis; enhanced cell production or anabolism; and rapid bone mineralization [19].

Many factors control phosphorus balance of which the most important is the amount reabsorbed by the renal tubules. The effect of hormones on phosphate balance is not fully elucidated. When vitamin D is given to a D-deficient subject, intestinal phosphate absorption increases. PTH reduces the renal reabsorption of phosphate, leading to hypophosphatemia, and calcitonin also increases phosphate excretion by the kidney [1].

2.3. MAGNESIUM

Magnesium, like potassium, is widely distributed in soft tissues and in the skeleton, wich contains up to 60% of the total body content [20]. Mg constitutes = 0,5% to 1% of

bone ash [21]. It is the second most abundant divalent intracellular cation and is involved in a large number of metabolic processes. The plasma magnesium level is remarkably constant and depends on rapid adaptation by the kidney. Thus a fall in magnesium intake is rapidly followed by a fall in urinary output and only later by hypomagnesaemia [1]. Homeostatic mechanism, such as those described for calcium, for selective mobilization of magnesium from the tissues do not appear to exist. The way in which the body protects against magnesium loss is by a reduction in urinary excretion, and only if the intake is very low for a long time or intestinal losses are high do the tissues lose magnesium significantly [1].

The remainder of Mg bone is an integral component of the hydroxyapatite lattice, wich may be relased during bone resoption [21]. Mg influences the formation and/or secretion of hormones that regulate skeletal homeostasis and the effect of these hormones on bone. Mg can also directly affect bone cell function as well as influence hydroxyapatite cristal formation and growth [20].

Mg modulates PTH secretion in a similar way to calcium: some *in vitro and in vivo* studies have demonstrated that acute elevations of Mg inhibit PTH-secretion, whereas and acute reduction stimulates PTH secretion [20, 22-26]. The inhibitory effects may be dependent on the extracellular calcium concentration, Mg acts through the Ca-sensing receptor, which mediates the control of extracellular calcium on PTH secretion [27].

Mg may also independently influence bone mineral formation. *In vivo* studies have demonstrated that as the Mg content on bone decreases, the hydroxyapatite crystal size increases, whereas high Mg content results is smaller crystals. Rats fed excess Mg have smaller mineral crystal in their bone than control pair-fed animals [28]. In contrast, Mg deficient rats have a significant increase in hydroxyapatite crystal size [29].

Mg may also have another indirect effect on crystallization by influencing both osteocalcin formation and osteocalcin binding to hydroxyapatite. The decrease in osteocalcin production as suggested by decreased serum and bone osteocalcin, in Mg depletion may influence mineralization [21].

2.4. ZINC

Zn is present in all the tissue and fluids on the body. The total body content has been calculated as 2-3g in an adult man, of which skeletal muscle accounts for approximately 6% and bone for about 30% [30].

The presence of a large amount of Zn in bone tissue suggests that this ion also plays an important role in the development of the skeletal system [31]. Zinc has a stimulatory effect on bone formation and mineralization, [32] whereas retardation of bone growth is a common finding in various conditions associated with Zn deficiency. Zn is required for the action of alkaline phosphatase (ALP) activity, this enzyme is mainly produced by osteoblasts whose major function is to provide calcium deposition in bone diaphysis. Zinc increases the half-life of ALP activity in human osteoblast-like cells [33]. The administration of both Zn or vitamin D3 produce a significant increase in bone ALP activity and DNA content, and the effect of vitamin D3 was synergistically enhanced by the simultaneous treatment with Zn [34]. The receptors for 1,25-dihydroxyvitamin D3 were shown to have two Zn-finger structures at the site of interaction with DNA [35]. One possible function of Zn is to potentiate the interaction of the 1,25-dihydroxyvitamin D3-receptorcomplex with DNA. Zinc directly activates aminoacyl-tRNA synthetase in osteoblastic cells, and it stimulates cellular proteinsynthesis. Moreover, Zn inhibits osteoclastic bone resorption by suppressing osteoclast-like cell formation from marrow cells. Zinc may act on the process of bone-resorbing factors induced by protein kinase C activation; these are involved in Ca^{2+} signaling in osteoclastic cells [32].

3. BIOCHEMICAL MARKERS OF BONE METABOLISM

3.1. BONE TURNOVER

The skeleton is continually renewed by a process called bone turnover, involving osteoclast-mediatedbone resorption, osteoblast-mediated bone formation, and remineralization of newly formed bone. Bone turnover, which takes place in bone multicellular units, is usually initiated by osteoclasts eroding a mineralized surface. When osteoclasts resorb bone, they secrete a mixture of acid and neutral proteases that act sequentially to degrade the collagen fibrils into fragments. Biochemical markers of bone resorption therefore include these collagen breakdown products such as hydroxyproline, hydroxylisine glycosides, and the piridinoline cross-links. Other markers are enzymes secreted by the osteoclast involved in the degradation of collagen type I, such as tartrate-resistant acid phosphatase and cathepsin K, and a non collagenous protein of the bone matrix, bone sialoprotein .

The initial step of bone resorption is followed by the recruitment of osteoblasts to the outer edge of the erosion cavity. The osteoblasts secrete new bone matrix (osteoid) that gradually fills in the resorption cavity. Biochemical markers of bone formation are

products of this osteoblastic activity. These osteoblast products include the amino and carboxy-terminal propeptides of type I collagen, osteocalcin, and bone-specific alkaline phosphatise. In addition to assigning bone turnovers markers specifically to the process of bone formation, certain markers appear to reflect different stages of these cellular activities. On the other hand, bone turnover markers cannot distinguish between diseases nor do they reflect disease-specific processes. The markers are also unable to distinguish between activities occurring at cortical or trabecular bone elements of bone .

3.2. MARKERS OF BONE FORMATION

3.2.1. Alkaline phosphatase

The enzyme alkaline phosphatase (ALP) belongs to a large group of proteins attached to the extracellular surface of cell membranes via a carboxy-terminal glycan-phosphatidyl-inositol anchor [36]. Because the most common sources of elevated ALP levels are liver and bone, a number of techniques have been developed to distinguish between bone and liver isoforms. There is a great deal of interindividual variation in ALP levels, but for one individual, values are stable over time. Bone ALP has a half-life of about 40 hours and like other glycoproteins is cleared by the liver [37]. Bone ALP are affected by age, gender, and hormonal status [38]. Fractures cause an increase that can last for at least one year [39, 40].

3.2.2. Osteocalcin

Osteocalcin is a small protein synthesized by mature osteoblasts, odontoblasts and chondrocytes. Osteocalcin is primarily deposited in the extracellular matrix of bone, but a small amount enters the blood. Serum osteocalcin is a sensitive and specific marker of osteoblastic activity and its serum level thus reflects the rate of bone formation [7-9]. There is some evidence that serum osteocalcin may also be derived from activities associated with bone resorption.

The main route of circulating osteocalcing catabolism is renal glomerular filtration and degradation. The level of osteocalcin are inversely correlated with creatine clearance [10]. Osteocalcin levels follow a circadian rhythm characterized by a decline during the morning to a low around noon, followed by a gradual rise that peaks after midnight [41]. Serum osteocalcin is increased after a fracture with levels remaining elevated for at least 3 months [42].

3.2.3. Procollagen peptides

The C propeptide (PICP), which is a trimeric globular glycoprotein, is stabilized by disulfide bonds and circulates as a single molecule [43]. It has a serum half-life of 6 to 8 minutes and is cleared by hepatic endothelial cells [44]. The procollagen type I amino-terminal propeptide (PINP) is a partly globular, partly helical (collagenous) protein. The intact molecule is cleared from the circulation by the scavenger receptor of liver endothelial cells [45]. Levels of PINP are higher in chronic kidney disease patients than age matched-controls and inversely, but not significantly, correlate with creatinine clearance [10].

Type I collagen is also a component of several soft tissues (fibrocartilage, tendon, skin, gingiva, intestine, heart valve, large vessels, and muscle) and this is the reason that there is a potential contribution to circulating procollagens from soft tissue synthesis of type I collagen, as is the case for almost all collagen type–related markers, including NTX and CTX. Nevertheless, because the rate of turnover of collagen in bone is faster than in other tissues, changes in procollagen concentrations are assumed to reflect changes primarily in bone collagen synthesis. Indeed, good correlations have been shown between PICP serum levels and the rate of bone formation [46, 47]. Similar to other markers, PICP and PINP demonstrate a circadian rhythm with peak values in the early morning hours and nadirs in the afternoon [48, 49]. Fractures increase levels of PINP significantly within 1 to 4 weeks, which remain elevated during at least 1 year [50, 51].

3.3. MARKERS OF BONE RESORPTION

3.3.1. Hidroxiproline

Hidroxiproline (OHP) is present in essentially all tissues and all genetic types of collagen. OHP is derived from the breakdown of collagen. The majority of the breakdown products are reabsorbed by the renal tubules and broken down in the liver, whereas only 10% is excreted in urine. OHP can never be reincorporated into newly synthesized collagen [52], but both collagen synthesis and tissue breakdown contribute to urinary hydroxyproline.

3.3.2. Collagen Cross- Links

3.3.2.1. Piridinoline (DPD) and Deoxypyridinoline (PYD)

Piridinolines act as mature cross-links in fibrillar collagens of all major connective tissues other than skin [53, 54]. This include type I collagen, which is present in bone,

dentin, ligaments, fascia, tendon, vascular walls, muscle and intestine (but absent from skin). In all tissues PYD predominates, with DPD being the minor component. However, DPD is most abundant in bone and dentin and is considered the mores specific bone marker because bone represents the major reservoir of total collagen in the body and turns over faster than most other connective tissues [53, 55, 56]. This conclusion is supported by the strong correlations between DPD and bone resorption rates as determined by radio-tracer kinetics [57].

3.3.2.2. Cross-Linked Telopeptides

- ***C-telopeptides:*** Two biochemical markers reflecting degradation of the C-terminal telopeptides of type I collagen have been described: CTX, which is released by cathepsinK cleavage of intact bone collagen, and ICTP, which is a larger fragment produced by matrix metalloproteinase (MMP) cleavage [58, 59].

ICTP is cleared from the body by the kidneyand circulating levels are reportedly elevated in the majority of patients with renal chronic failure [60]. Fractures have been reported to increase ICTP levels by 73%, wich may remain elevated for one year.

The cross-linked C-terminal telopeptide of type I collagen, or CTX-I, is a sensitive but also rather variable marker of bone resorption. Sistemic CTX is excreted by the kidney as well as metabolized with a renal excretion of about 44% [61]. CTX depends on renal [62] and liver function [63, 64]. Urine and serum CTX show considerable diurnal variation with a maximum of about 5 am and a minimum at about 2 pm. Moreover, a significant influence of fasting have been described [65].

- NTX

Another highly sensitive marker of bone resorption is cross-linked N-terminal telopeptide of type I collagen, also referred to as NTX. Serum levels of NTX as well as renal excretion of NTX are related to renal function [66]. Both serum and urine NTX levels follow a pattern of diurnal variation, although this seems less pronounced for serum NTX [67, 68]. NTX was slightly decreased one year after an ankle fracture [50], but increased slightly after a forearm fracture [51].

3.3.3. Galactosyl Hidroxylisine

Hidroxylisine, another modified amino acid particular to collagens, is glycosylated to varying degrees depending on the tissue type [69]. Two glycosides are formed,

Galactosyl Hidroxylisine (Gal-Hyl) and *Gucosyl Galactosyl Hidroxylisine* (Glc-Gal-Hyl). Because of tissue-specific differences, Gal-Hyl is considered to be relatively specific to bone collagen degradation [70]. Glycosylated hydroxylisine residues appear not to be reused or catabolised when collagen is degraded. Furthermore they do not appear to be absorbed in significant levels from a normal diet [69].

3.3.4. Tartrate- Resistant Acid Phosphatase

During bone resorption, osteoclasts secrete protons and enzymes into the space between the ruffled border of the osteoclast and the surface of the bone. The enzyme Tartrate-Resistant Acid Phosphatase (TRAcP) have been identified in both the ruffled border of the osteoclast membrane and the secretions in the resorptive space [71]. Six isoenzymes (types 0 to 5) of acid phosphatase have been identified in human tissues [72]. TRAcP5b is predominant in bone and shows a relatively small but significant diurnal variability (14%) and negligible effect of food intake (2%) [73]. Because TRAcP5b does not accumulate in patients with end-stage renal disease, the marker does not seem to be cleared directly by the kidney [73, 74].

3.3.5. Bone Sialoprotein

Bone Sialoprotein (BSP) is an acidic, phosphorylated glycoprotein that is syhthesized by osteoblasts and osteoclastic-like cells in culture. BSP stimulates hydroxyapatite formation *in vitro.* It has been suggested that BSP may play a role in angiogenesis associated with bone formation, tumor growth and metastasis [75]. A small amount of BSP is found in the circulation and as such is a potential marker of bone turnover [76, 77].

3.3.6. Cathepsin K

The enzyme cathepsin K is a member of the cysteine protease family that, unlike other cathepsins, have the unique hability to cleave both helical and telopeptide regions of collagen type I [78-80]. The enzyme is located in osteoclasts and is secreted into both resorption lacunae for extracellular collagen degradation [81].

4. FACTORS INFLUENCING BONE REMODELING

4.1. BONE EFFECTS OF THE DIETARY AMOUNT OF PROTEIN

Nowadays, the effects of HP diets consumption on bone health status still appear to be a controversial issue with authors either reporting a deleterious influence in healthy humans [82, 83] and rats [84, 85], or describing no adverse or even a protective effect derived of HP supplements in rats [86-89] and humans of different ages [90, 91].

4.1.1 A high-protein diet may negatively affect bone health

Traditionally, high dietary protein intake was thought to be harmful for bone health. Already by 1968, Wachman and Bernstein [92] hypothesized that the increased incidence of osteoporosis with aging was the result of ‘‘the life-long utilisation of the buffering capacity of the basic salts of bone for the constant assault against pH homeostasis’’ since the Western meat diets contain ‘‘acid ash’’. It has long been hypothesized that diets high in acid cause a low-grade metabolic acidosis and mobilize base from the skeleton, thereby contributing to bone loss with aging. Therefore, urinary calcium excretion is strongly related to net renal acid excretion [93, 94] and has found to be clearly higher in rodents and humans that consumed a HP diet. The catabolism of dietary protein generates ammonium ion and sulphates from sulphur-containing amino acids. In general, it is considered that protein is a net acid-producing substance and thus a net negative risk factor for bone dissolution. According to the ‘acid-ash hypothesis’ above described, an excess of acid load would be neutralized by the release of bicarbonate ions from the bone matrix, a mechanism that is accompanied by a loss of Na^+, K^+ and a small amount of calcium^{2+} [95], and consequently, the increase in bone resorption is reflected by the increase in urinary calcium excretion [96-98]. The acid load would also decrease osteoblastic activity and increase osteoclastic activity, resulting in net bone resorption with mobilization of calcium [99, 100]. However, no convincing experimental data support this theory. The acidogenic role of dietary protein remains being a matter of debate, as it is well established that other food components have a clear impact on acid-base balance [96, 101].

Most of evidence about the negative effect of HP diets on bone health came from retrospective epidemiological studies based on hip fracture incidence in postmenopausal women from different countries [102, 103]. In such studies, the authors have observed that the highest rate of hip fractures occurred in industrialized Western countries, which

have the highest animal protein intake. However, several obvious limitations to these studies has been annotated by Bonjour [103]. First, countries with the highest incidence of hip fractures are also those with the longest life expectancy, which is an important determinant of the risk of osteoporotic fracture [104]. Second, protein intake was estimated from the whole population but not for the specific studied group, and finally, interethnic differences on risk of osteoporotic fracture are well known and may be attributable to many factors such as bone structure, genotype or lifestyle [22, 23].

It is unclear whether bone loss is directly the result of acid load itself [105], or occurs secondarily from release of bone-resorbing prostaglandins or cytokines. In fact, bone loss has been attributed to elaborating bone-resorbing cytokines [105]. Moreover, acid load from animal protein excess has been found to inhibit osteoblastic collagen synthesis in vitro [99].

Results on changes in urinary hydroxyproline, a marker of collagen metabolism, in response to HP diets are controversial, with some studies reporting elevation of urinary excretion of hydroxyproline with HP diets [36, 37] whereas other did not observe any change [106, 107].

Lower urinary pH is also consistently observed for subjects consuming HP diets, especially from animal sources. A decrease in urinary pH, together with the consequent hypocitraturia and hypercalciuria promoted by a HP diet intake, are recognized risk factors for kidney stone formation [87, 108].

Furthermore, urine acidification is also a characteristic of visceral obesity and the metabolic syndrome, and thus HP diets may be associated with various metabolic abnormalities in visceral obesity [109].

4.1.2. A high-protein diet may positively affect bone health

There is recent substantial literature supporting the beneficial effects of HP consumption on skeletal metabolism when such protein is consumed together with adequate calcium, potassium, and other minerals, regardless of the source of protein [93, 94]. In fact, the majority of recent observational studies support a positive association between protein intake and bone health. There are several epidemiological studies, both cross-sectional and longitudinal, that have reported a positive association between dietary protein and bone [10, 40]. These studies reveal that individuals whose consume the most dietary protein have the highest BMD. Accordingly, prospective studies have

observed that individuals with the highest protein intake have the slowest rate of bone loss in elderly men and women [91, 110].

Protein level seems to be crucial for development of bone and muscle mass. Several epidemiological and clinical studies point to a healthy effect of protein intakes above the current Recommended Dietary Allowance (RDA) (0.8 g/kg per day) for adults aged 19 and older [111]. Moderate protein diets of 1.0 to 1.5 g/kg per day were shown to be associated to normal calcium homeostasis without altering bone metabolism [112]. Although this level is approximately twice the current RDA, it is still within the acceptable range of intake (10–35% of total calories) [111]. In fact, some recent studies [113] have demonstrated that the recommended daily intakes of protein 0.8 g/kg/day could be undervalued. The mean and population safe requirements were determined to be 0.93 and 1.2 g/kg/d and are 41% and 50 %, respectively higher than the current RDA recommendations [114].

The Saskatchewan Paediatric Bone Mineral Accrual Study [115] highlighted the importance of dietary protein intake during adolescence, when peak bone mass is being accrued. Peak bone mass is obtained somewhere between the ages of 16 and 18 and remains relatively stable until 35 to 40 years, after which bone mass, like muscle mass, begins to decline [116]. Maximizing peak bone mass is therefore an important way to prevent osteoporosis. Although calcium and vitamin D are essential for bone health, recent evidence supports the hypothesis that dietary protein is also critical for bone health and fracture reduction [103]. Bone is approximately 50% protein matrix, so it is not surprising that dietary protein is an essential nutrient for the development of maximum peak bone mass, although recent evidence also suggests that dietary protein could have an important role in skeletal health throughout adulthood and elderly[91, 112]. In concordance, Geinoz et al. [117] and Hannan et al. [110] have reported that HP diets were associated with higher BMD in femoral neck in elderly men and women. However, Arjmandi et al. [118] observed in postmenopausal women that the daily consumption of 25g protein for one year irrespective of the source of protein resulted in no significant changes on hip BMD and bone mineral content. Similarly, in a longitudinal study including 540 premenopausal women, the authors found no adverse effect of increased protein intakes on BMD [119]. In their systematic review, Darling et al. [120] observed that a large majority of the cross-sectional surveys or cohort studies reviewed reported either no association or a beneficial association between proteins and

BMD, and only one survey found a negative correlation between proteins and bone mineral content. The authors concluded that HP, if not significantly favourable, are at least not detrimental for bone density.

Epidemiological data currently support the hypothesis that a habitually HP intake promotes bone health [91, 121], but controlled intervention studies with BMD and fracture rates as the primary outcome variables are needed. In fact, there is evidence that the anabolic response of muscle to dietary protein is attenuated in elderly people, and as a result, the amount of protein needed to achieve anabolism is greater [111].

Nonetheless, a counter-argument has been made that protein-associated hypercalciuria is due to enhanced intestinal calcium absorption and not the breakdown of bone [122, 123]. Moreover, despite an excess of dietary protein from either animal or plant proteins may be detrimental to bone health, its effect will be modified by other nutrients [93]. Indeed, Pye *et al*. [124], found that lower body weight, fat mass and higher lean body mass were associated with a high mixed protein consumption by rats, but did not find any deleterious effect on bone. The authors concluded that a mixed HP diet containing adequate calcium levels and up to 35% of energy can be deemed safe for long-term bone health.

Among the mechanisms involved on the beneficial effect of protein on bone health may be implicated the IGF-1. Insulin-like growth factor I is one of the major regulators of bone metabolism and can act as a systemic and local regulator of osteoblastic function [125, 126] and as a coupling factor in bone remodelling by activating both bone resorption and bone formation [127]. Dietary protein increases circulating IGF-1, which has anabolic effects on muscle and bone. This effect has been observed in both animal and human studies [128-130].

There is some evidence that the beneficial effect of protein intake on bone mineral mass is better when supplies of calcium, potassium and vitamin D are adequate [93, 94, 129, 131, 132]. The positive effects of dietary protein intake on bone health seem to be dependent, at least in part, on calcium intake [133]. In this regard, Heaney et al. [134] have shown that HP diets have adverse effects on bone health only if dietary calcium and Potassium intakes were not at the recommended levels. Furthermore, maintenance of adequate bone strength and density with aging is highly dependent on the maintenance of adequate muscle mass and muscle mass is in turn dependent on

adequate intake of high-quality protein [135, 136]. In fact, bone remodeling involves the synthesis of new protein matrix and requires an ongoing supply of fresh dietary protein if bone removed during resorption is to be replaced [137, 138].

Recent studies suggest that dietary protein works synergistically with calcium to improve calcium retention and bone metabolism [137-140]. Isotopic studies in humans have also demonstrated greater calcium retention and absorption by individuals consuming HP diets, particularly when the calcium content of the diet was limiting [140]. Thus, HP diet does not seem to lead to calcium bone loss, and the role of protein seems to be complex and probably dependent on other dietary factors and the presence of other nutrients in the diet [93, 133]. However, HP diets usually contain low amounts of fruits and vegetables, which yet appear to be beneficial to bone health [103]. It appears reasonable to avoid very HP diets when associated with low calcium intake [141]. Nevertheless, the interaction between dietary protein and other components in a mixed diet, such as calcium intake and vegetables and fruit to neutralize acid, are important and may determine whether HP diets are beneficial to bone health [102, 133, 139].

4.2. INFLUENCE OF THE SOURCE OF PROTEIN ON BONE STATUS

4.2.1. Animal versus vegetal protein intake

Many studies have also gone toward researching which might be the best source of protein for bone health in both animals [142] and humans [82] studies. Attempts to demonstrate the superiority or inferiority of animal versus vegetable protein on bone health has been confounded by the fact that communities consuming more vegetable protein tend to also consume more fruit, vegetables, and potassium, which tends be more alkaline, with some bone beneficial effects [143]. On the other hand, it has been postulated that the sulfur content of animal proteins is greater than that of vegetal proteins. This argument does not hold when considering straightforward chemical analysis of the sulfur content of different proteins [103, 133]. Therefore, the division into animal and vegetable protein sources has been made as a crude index of the acid load of protein [144].

Sellmeyer et al. followed 1,035 women more than 65 years of age prospectively for 7 years [82]. Protein intake, whether of vegetable or animal origin, was noted from a food frequency questionnaire. The authors found that women with a higher ratio of animal to

vegetable protein had greater bone loss at the femoral neck and greater risk of validated hip fracture after adjustment for confounding factors including calcium intake. Similarly, in the Rancho Bernardo Study [102], 572 women and 388 men aged 55–92 years in Rancho Bernardo, California, were followed for 4 years. The authors observed a negative relationship of increased protein intake and decreased BMD just for vegetable protein [102].

The effect of varying amounts of dietary calcium is also an issue. Hence, epidemiological studies can tend to favour vegetable protein. For example, a global analysis was performed by recording hip fracture rates in 100,000 women older than 50 years of age from 33 different countries [145]. Those 11 countries in the lowest tertile of hip fracture rates had the lowest animal protein consumption, with vegetable protein intake exceeding animal protein. Of the 11 countries in the highest risk tertile for hip fractures, animal protein consumption exceeded vegetable protein intake in 10 countries. In the EPIC Potsdam Study [146], the relationship between bone health and diet, particularly animal and vegetable protein and their interaction with calcium was studied. The apparent detrimental effect of animal protein on bone was attenuated in those subjects with a high calcium intake. To note is that there was a significant interaction between calcium and animal protein but not calcium and vegetable protein.

Among the different effects of various proteins on bone status, whey and soy protein have been highly studied and compared. Whey is the liquid remaining after milk has been curdled and strained to remove the caseins (curds). It contains proteins, lactose, vitamins, minerals, and traces of fat. Whey protein represents 20% of the total protein content of milk and has benn reported to have utility in many different applications ranging from effects on bone, muscle, blood, brain, pancreas, immune, cancer, infection, metabolism, wound healing, learning, and aging [147]. Soy protein is a vegetable high quality protein, with a protein digestibility corrected amino acid score of 1; it also has a high arginine/lysine ratio, which is associated with lower insulin secretion compared to animal protein. Afterwards, soy protein contains isoflavones, which act as weak estrogens, inhibiting tyrosine kinase-dependent signal transduction processes and functioning as cellular antioxidants [148, 149].

4.2.2. Whey protein and bone status

As mentioned above, the effect of milk whey protein supplementation (particularly its basic protein fraction) on bone health has been deeply studied. The Copenhagen Cohort Study was a cross-sectional study performed on 63 seventeen-year-old girls and 46 boys [150]. A 7-day diet analysis was performed, and bone mineral content and serum markers of bone turnover, as well as IGF-1 were measured. Both total and milk (0.3g/kg) protein intake, but not meat protein intake (0.4g/kg), was positively associated with size-adjusted bone mineral content. The positive association between milk protein intake and size-adjusted bone mineral content remained significant after controlling for calcium intake, physical activity, and energy, and did not seem to be mediated via IGF-1.

Some studies were performed in Japan to test the effects of Snow Brand Milk Products [151, 152]. The authors studied the effect of doses ranged 40–300 mg of milk basic protein (MBP) supplementation for 6 months in healthy women, postmenopausal women, and healthy men. They observed a suppression of bone resorptive markers such as urinary cross-linked N-telopeptides of type I collagen/creatinine and deoxypyridinoline/creatinine and increased markers of bone formation like osteocalcin in all groups [151, 152]. They also demonstrated a modest effect on BMD in calcaneus, lumbar spine, and radius. However, not all studies have shown a convincing benefit of MBP [102]. In the study by Zou et al. [142], 84 healthy young women were divided into three groups receiving placebo, whole milk, or milk containing 40mg of MBP for 8 months. Total BMD significantly increased compared to the baseline values in all groups. However, no significant difference on the mean rate of gain of total BMD was observed among the MBP group, the whole milk group, and the control group [102].
Whey protein has been postulated to improve bone health in vitro as well as in rodent models [153, 154]. Du et al. [154] examined the effects of whey basic protein on bone metabolism of Sprague-Dawley rats and healthy mid-aged women for 90 days. The authors observed that whey protein should enhance the BMD of the rat's femur, however, no obvious effect was detected in the human group. More recently, Xu et al. [153] analyzed the effects of whey protein on osteoblasts in vitro and they observed that whey protein stimulated the proliferation and differentiation of osteoblasts cultured at different concentrations of whey protein. The levels of osteocalcin and IGF-I in the culture medium also increased. Real-time reverse transcription-PCR results showed that

the mRNA expression of osteoprotegerin and receptor activator of nuclear factor-kappa B ligand increased in the cells in a dose-dependent manner. The authors concluded that active component in the whey protein plays an important role in bone formation and a potential therapeutic role in osteoporosis by activating osteoblasts [153].

4.2.3.. Soy protein and bone status

In general, animal studies have shown that isoflavones in the context of soy protein have positive effects on BMD [57-59]. The findings of clinical trials have ranged from no significant changes [155-157] or a slight increase [63, 64] on BMD. Nevertheless, the bone protective effects of soy and/or its isoflavones are at best inconclusive. Regarding bone effects, a number of studies have been conducted in female experimental animals and post-menopausal women using soy products or soy-associated isoflavones as an alternative to classic estrogens to restore bone loss stemming from sex steroid deficiency [158]. Soy protein is notable for its low fat content and for containing phytochemicals such as isoflavones but also saponins, and phytic acid. Phytates are naturally occurring compounds particularly found in whole grains. Despite concern that they may reduce calcium absorption, there is some evidence that they are beneficial for a better bone health [143].

Some authors do not support a bone protective role for soy protein and its isoflavones [118, 157]. Whether higher amount of soy protein and/or its isoflavones can reverse bone loss remains to be illustrated. In the subset of the Shanghai Women's Health Study [159], women in the lowest quintile of soy intake actually had a relative risk of 0.63 for hip fracture compared to the highest quintile. Gallagher et al. [157] supplemented the diets of postmenopausal women for 9 months with 40g soy protein delivering three levels of isoflavones (0, 52, and 96 mg) and all three groups experienced bone loss. In a recent study, performed by Zhoe at al. [160] in premenopausal women, incorporating approximately 19 grams of soy protein from soy foods for 10 weeks had no significant changes in bone resorption, thyroid-stimulating hormone, or free thyroxine [160]. Similarly, BMD was not significantly affected after supplementing postmenopausal women with 150 mg isoflavones twice daily for 6 months [161] or with 99 mg isoflavones/d for 12 months [156]. Nonetheless, higher doses of soy protein with varying levels of isoflavones have not consistently shown to exert beneficial effects on bone.

Conversely, other studies have found a clear positive effect of soy protein on bone health. Soy foods have been largely associated with improved markers of bone health. In the study by Zhang et al. [162] soy protein isolate and 17beta-estradiol had different effects on bone turnover prior to puberty in rats. Approximately half of the genes were regulated in the same direction by17beta-estradiol or soy protein isolate, but in combination, soy protein isolate blocked the estrogen effects and returns the profile towards control levels [162]. In the cross-sectional studies by Ho et al. [163] and Horiuchi et al. [164], respectively, in postmenopausal women, increased dietary soy protein was associated with increased BMD at hip, total body, and spine, especially when high versus low isoflavone concentration. Six-month studies by Potter et al. [63] and Alekel et al. [155] also reported positive effects of soy protein supplementation on BMD. Under an animal model, Setchell et al. [165] provided data that clearly indicate that consumption of dietary soy protein isolate promotes bone growth in both intact animals and in the absence of estrogens. In sharp contrast, soy protein increased serum bone formation markers, but decreased bone resorption markers supporting the hypothesis that soy protein influence the rodent bone in a significantly different manner than estradiol. This was also supported by their in ex vivo osteoblast differentiation data. The ability of soy protein isolate to affect bone formation and osteoblast activity indicates that it possesses specific bone anabolic properties. The bioactive soy protein isolate components responsible for these effects remain to be identified.

Other studies using soy protein at similar or higher doses of isoflavones have concurred to mixed results. Wangen et al. [74] provided dietary isoflavones at 65 mg/d and 132 mg/d and found no clinically relevant effects on bone biomarkers or hormones. Alekel et al. [155] suggested that 40g soy protein supplementation with 80mg isoflavones was able to attenuate bone loss from lumbar spine but women still lost 0.2% BMD in six months. Potter et al. [63] showed that 40g of soy protein containing 90 mg isoflavones was able to attenuate loss in lumbar spine BMD, however, the same amount of protein with 56 mg isoflavones content had no effect.

Roughead et al. [166] suggested that soy protein may be more favourable at doses above 40 g/day and in the presence of calcium supplementation (e.g., 650–1,400 mg in previous studies), such as in some of the studies already discussed [102, 155, 167]. Accordingly, in the study by Spence et al. [168], the authors cannot exclude the possibility that soy isoflavones increase calcium retention at high doses or that soy

isoflavones are site specific or more effective immediately after menopause, when bone turnover is higher.

Despite that observational studies have linked high soy protein consumption with lower osteoporotic fracture risk, but the treatment effect of soy protein on bone density and microstructure remains controversial in healthy populations. In the last years, much attention has been focused on the phytoestrogens contained in soy protein. Furthermore, recent research suggests that the efficacy of soy protein on bone depends on the ability of the body to convert the soy isoflavone daidzein to eqoul, a potent estrogenic metabolite [169]. Only 20–35% of the population has been reported to have the ability to metabolize daidzein to eqoul via intestinal microflora [169], which may partially explain the differential responses to soy protein interventions in the context of bone health.

When comparing both sources of protein (whey vs soy) whey protein seems to have less effect than soy protein on bone protection, especially in menopausal women. Chen et al. [170] compared the effect of soy and whey protein hydrolizate and rice protein isolate on bone in both intact and ovariectomized rapidly growing female rats. They observed that soy protein had superior anabolic effects to enhance bone mineral density and bone mineral content in intact young female rats and more than other protein source in treatment of ovariectomized-induced osteopenia in female rat models [170]. Finally, a study involving men, no effects of soy protein on BMD was observed when compared to milk-derived proteins [171].

4.3. INFLUENCE OF THE EXERCISE ON BONE STATUS

4.3.1. Effect of nutrition and exercise on bone mass development

The benefits of exercise on bone health are highly contrasted by numerous studies in humans and other animals. Resistance training benefits on bone mineral content have been largely demonstrated [172]. In fact, in humans, physical activity has a positive effect on bone mass and microstructure in adolescents [173], adults [174, 175], and the elderly [176, 177]. In animal models, forced exercise on a treadmill or jumping has also shown a positive effect on bone strength [178, 179]. However, since forced-exercise models may be problematic due to stress, voluntary wheel running may be an alternative model. A few studies have indicated that voluntary exercise can improve bone properties through bone modeling or remodeling [142, 143]. Overall, though, data

on the effect of voluntary exercise on bone in animal models remain limited. Both diet and exercise should be considered when optimizing the effects on body composition and bone, even though the underlying mechanisms remain partly unknown [180].

Aparicio et al. (2011) [181] showed resistance training was also effective at enhancing bone mineral content, as measured by femur ash weight (relative to dry femur weight). These effects were observed after 3 months of intervention, suggesting a mid- to long-term effect. In the study of Bennell et al. (2000) [182] performed in rats, the authors did not appreciate differences in the bone of rats developing a similar training protocol. On the other hand, Burr et al. (2002) [183] reported that short periods of interrupted resistance training, with rest periods between them, were a more effective osteogenic stimulus than a single sustained session.

Bass et al. suggested that exercise during growth seems to increase the BMD peak by between 10% and 20% in the loading bones of active adolescents compared with sedentary controls [148]. It is also likely that exercise during this period acts in a synergic way, with the growth-related bone development leading to a higher bone mass at the end of the pubertal period [184, 185].

Bone seems to adapt to the level of exercise intensity required [186] depending on the mechanical stress generated by exercise. Therefore, the final effects of exercise on bones depend on the type, intensity, and duration of the stimulus. Although the most suitable sporting activities remain unknown, participation in weight-bearing activities generating high ground reaction forces, mainly if they include jumps, sprints, and rapid changes of directions, seem to have the most evident osteogenic effect during growth [152, 153].

Body mass and exercise evidently act on bone through altered mechanical loading. Mechanical loading modifies bone structure or density by stimulating signaling pathways that regulate bone formation and resorption. Furthermore, abundant evidence suggests that adipose tissue as an endocrinal organ affects bone metabolism through secreted adipokines [187-189].

4.3.2. Mechanism of action

It is clear that bone cells are able to sense their mechanical environment via a number of proposed mechanism including direct matrix deformations, pressure and transient

pressure waves, accelerations, fluid shear stresses, fluid drag forces, or even dynamic electric fields as interstitial fluid flows past garged bone crystals. It is much less clear, however, which of the cells within a bone are the key sensory elements in mechanotransduction [190].

Mechanical responses of osteoprogenitor cells, included estromal cells, osteoblasts and osteocytes have all been documented without identifying the critical responding cell: for example, exposure to microgravity results in a decreased number ofosteoblasts, but the cell senses and respons to the loss of gravity are not well know [190]. There are the following possible options: the undifferentiated mesenchymal precursor, the differentiated osteoblast or the osteocyte. Bone marrow stromal cell change their proliferation rate and gene expression patterns in response to mechanical stimulation [191] and alter lineage selection as well [138]. With respect to the osteoclasts and bone resrption: stromal cell expression of the osteoclstogenic factor, RANKL, is sensitive to mechanical force [192], suggesting that the number of osteoclasts itself has also been shown to respond to mechanical force might limit bone resorption [193].

Other cells present in bone, such as endothelial and smooth muscle cells in the prenetating vasculature, might also contribute to the skeleton´s adaptative response to loading. Endotelial cells respond to shear stress and tensile strain generated by increased hear rate during exercise, by producing nitric oxide [194]. Increased vascular release of nitric oxid is likely to regulate bone cell response: nitric oxide has pleiotropic effects in bone cells, and potently decreased resorption through decreases osteoclst formation and activity [141]. But bone stromal cells also release nitric oxide as a result of mechanical input [195] providing a secondary cell target for mechanical induction of this freely diffusible antiresorptive agent.

The majority of cells in cortical bone are osteocytes, and owing to their pervasive, three-dimensional distribution throughout both trabecular and cortical bone, these cells are potentially well place to sense the magnitude and direction mechanical strain within the tissue. Osteocytes although enclosed in calcified tissue, are interconnected through a nertwork of cannaliculi through which cells cast long cell processes. Osteocytes respond to strain in vivo as shown by increased glucose-6-phosphatase dehydrogense activity [196] or earlier response of c-fos mRNA [197] after loading. As well, unloading causes osteopontin expression in osteocytes [165].

The cellular response to mechanical stimuli are: fluid flow, prostanoids and nitric-oxide, which stimulate bone cell proliferation and matrix formation [198].

The effects of fluid flow: Mechanical loading of bone not only deforms the bone tissue, but also engenders movement of extracellular fluid through the bone´s lacuna-canalicular system.Such fluid flow may stimulate bone cells via streaming potentials, whall shear stress or chemo-trnsport related effects [167-169].
It is more than probable that bone cells respond to more than one component of their mechanical environment. This is suggested strongly by the ability of mechanical strain as well as fluid flow stimuliti to promote bone cell activity [168, 170-173]. Since in vivo loading is always accompanied by fluid flow, bone cells may respond to changes in their strain environment via and integrated assessment og changes in multiple parameters associated with both fluid flow and physical deformation. Such a response might allow for a more structurally relevant remodelling response in different anatomic locations or in bone with different architectural (cortical vs. cancellous) properties [174, 175].

Prostanoids and Nitric Oxide: the osteocytes have been demonstrated to be mechanoresponsive and, within the range of strains they encounter in vivo, to produce significantly higher levels of the signalling molecules PGE2 and PGI2 than osteoblasts in response to pulsating fluid flow [176-178].

4.3.3. More effects of exercise and nutrition on bone status

High protein intake may be appropriate for some athletes, but there are potential negative consequences that must be carefully considered before adopting such a diet. In particular, care must be taken to ensure that there is sufficient intake of other nutrients to support the training load [199].

Moreover, increased calciuria does not necessarily relate to bone loss. In fact, there is evidence that increased protein intake leads to improved bone health [47, 84]. Certainly, synthesis of bone collagen is increased by protein ingestion [200]. Given that many modes of exercise, e.g. resistance exercise, walking, running, etc., are stimulatory for

increased bone mineralisation, as well as the issues mentioned above, it seems unlikely that most high-protein diets will lead to bone problems in regular exercisers.

Previous animal studies have demonstrated prevention of bone loss and reduction of cardiovascular risk with a combination of soy isoflavones and exercise [201, 202]. Therefore, Tomayko et al. [203] evaluated the effectiveness of a soy protein diet and exercise training, alone and in combination, on vascular and bone measures in a mouse model of moderate to severe renal insufficiency.
The primary findings in that study were that both a soy protein diet and exercise training significantly increased relative bone volume and improved bone microarchitecture in a mouse model of disordered lipid metabolism and surgically induced renal insufficiency. The effect tended to be greatest when these interventions were administered in combination. Although the interaction effect was not significant for any of the bone measures (with the exception of total volume as a component of relative bone volume, or BV/TV), the Soy/Exercise combination group tended to have more favorable bone variables when compared to the other treatment groups.

Several studies have found a synergistic effect of phytoestrogens and exercise on improving bone parameters in ovariectomized mice, rats and premenopausal women [204]. Exercise is known to stimulate the estrogen receptor on bone, and this action may contribute to the enhanced bone microarchitecture seen in the soy and exercise combination group. Oh et al. [202] have suggested soy isoflavone supplementation protects against exercise-induced oxidative stress, which may explain why the benefits demonstrated in the Tomayko´s study (2011) [203] were more pronounced in the combination group compared to exercise training alone.

It has also been described a joint action of calcium intake and physical activity on bone mass. Increases in BMC and BMD in the bone areas experiencing tension have been observed, notably in high-performance activities with impacts or a high volume of training [147-150]. Therefore, it seems logical, as has been indicated in several studies [151-154], that the combination of physical activity and calcium intake may be more effective in increasing bone mass than either calcium intake or physical activity alone [205].

Nevertheless, it seems that a minimum calcium intake of approximately 1000 mg/day is required to make effective these combined inputs [206], as exercise without sufficient calcium supply would not increase the bone mass in adolescents [207].

Therefore, it seems that there is a very important interaction between the mechanic requirement of physical activity and the availability of calcium (and possibly the availability of other nutrients) to reach the greatest bone mass increases. Specker and Binkley [208] showed that both exercise and calcium supplementation had an effect on bones, and they found an interaction between calcium intake and exercise, translated into changes in leg BMC by dual-energy X-ray absorptiometry (DXA) and geometry by quantitative computed tomography (QCT). However, while a calcium supplementation effect disappears, exercise effects were still significant one year after the intervention [209].

4.3.4. Bone mineral content and physical training

There are also few studies regarding the effects of physical training on the bone mineral density in young mice. The studies on bone minerals were focused on Ca, due to the intensified interest on osteoporosis in aging, especially for the aged animals. There are a few studies concerning the Mg content of bone, as mentioned above, but none of them addresses Zn content for the growing mice under exercise [210].

However, magnesium and zinc play an important role in physical performance since the cofactors of enzymes are involved in both energy production (magnesium and zinc) and the antioxidant defence system (zinc) [211]. Different exercise modes have different effects on growth and bone mass [162, 163]

4.3.5. Exercise and calcium intake

If exercise is combined with increased calcium intake or supplementation, the benefits on bone mass seem to be greater. The latest evidence coming from randomized controlled trials shows that there is interaction between calcium supplementation and exercise, resulting in important changes not only in BMC but also in the structural properties of bones [212].

4.4. ANABOLIC ANDROGENIC STEROIDS

4.4.1. Global effects of anabolic androgenic steroids on bone

Anabolic androgenic steroid (AAS) use is widely spread in both professional and recreational sports, and the potential health risks are of great concern [213]. Despite the widespread usage of AAS, the knowledge of the long-term effects and side effects of these substances at high doses is incomplete. A common sought after effect of AAS is changes in body composition, with increased muscle mass and decreased fat mass but the studies that have investigated the effect of AAS on body composition in strength athletes have yielded ambiguous results [214-219].

The potential effects of AAS in different tissues are modulated at the cellular level by tissue-specific steroid converting enzymes. There is accumulating evidence that in a range of tissues the eventual cellular effects of testosterone may not be the result of direct action of testosterone, but may also reflect the effects of sex steroid metabolites formed as a consequence of local enzyme activities. The most important testosterone metabolites likely to influence bone are 5 α- DHT (the result of a 5α reduction of testosterone) and estradiol (formed by aromatization of testosterone) [220]. In adipose tissue, that is, testosterone is converted by aromatase to estrogen, whereas in human skeletal muscle, testosterone binds directly to androgen receptors [221]. Modest levels of aromatase activity have been reported in bone from mixed cell populations, derived from both sexes [222-224] and from osteoblastic cell lines [225]. There is little to non detectable aromatase mRNA expression in osteoclasts, or in cortical bone in mice [178]. It is clear that androgens have effects on skeletal development [226] and on the maintenance of bone mass, stimulating bone formation and inhibiting bone resorption, likely through multiple mechanisms that involve both androgen and estrogen-mediated processes [220].

In addition to endogenous steroid metabolites, there are also a variety of drugs with androgenic activity. These include anabolic steroids that can bind androgens receptors albeit lower affinity than testosterone [180]. The available scientific literature describes that short-term administration of these drugs by athletes can increase strength and body weight. Strength gains of about 5–20% of the initial strength and increments of 2–5kg body weight, that may be attributed to an increase of the lean body mass, have been observed. A reduction of fat mass does not seem to occur. Although AAS administration may affect erythropoiesis and blood haemoglobin concentrations, no

effect on endurance performance was observed [227]. The main untoward effects of short- and long-term AAS abuse that male athletes most often self-report are an increase in sexual drive, the occurrence of acne vulgaris, increased body hair and increment of aggressive behaviour. AAS administration will disturb the regular endogenous production of testosterone and gonadotrophins that may persist for months after drug withdrawal. Cardiovascular risk factors may undergo deleterious alterations, including elevation of blood pressure and depression of serum high-density lipoprotein (HDL), and cholesterol levels. In studies of athletes, AAS were not found to damage the liver [227]. Psyche and behaviour seem to be strongly affected by AAS. Generally, AAS seem to induce increments of aggression and hostility. Many other adverse effects have been associated with AAS misuse, including disturbance of endocrine and immune function, alterations of sebaceous system and skin, changes of haemostatic system and urogenital tract. AAS stimulate erythropoietin synthesis and red cell production as well as bone formation but counteract bone breakdown [227].

Furthermore, AAS are used to treat disturbances of nitrogen balance and muscular development and several other non-endocrine diseases, including several forms of anaemia, hereditary angioneurotic oedema, breast carcinoma and osteoporosis [227].

In a number of case studies, AAS have been reported to affect the musculo-skeletal system by causing bone fractures, tendon pathology [182-185] and rhabdomyolysis [208, 209].

4.4.2. Specific effects of anabolic androgenic steroids on bone

It has been demonstrate that androgens are important in the maintenance of a healthy skeleton, and have been shown to stimulate bone formation in the periosteum [178, 188] but reduce formation on the endosteal surface in cortical and in trabecular bone [210], thus, androgen increases radial growth in cortical bone through periosteal apposition. Evidence suggests that androgens act directly on the osteoblasts, and it appears that exposure time is an important variable. The effect of androgen on osteoblasts have been shown to be biphasic, with enhancement following short or transient treatment but significant inhibition following longer treatment [211, 212]. As an additional component of control of osteoblast abundance, it is important to consider the process of programmed cell death, or apoptosis [228], in fact, chronic DHT treatment has been shown to result in an enhanced osteoblast apoptosis *in vitro* in both proliferating osteoblastic at day 5, and in mature osteocytic mineralizing cultures at day 29 [229].

Apoptotic cell death could thus be important in making room for new bone formation and matrix deposition, which may have clinical significance by influencing bone homeostasis and bone mineral density [215]. Thus, mounting evidence suggests that chronic androgen treatment increases neither osteoblast number nor viability in the mature bone compartment. It is interesting to speculate that, given strong androgen-mediated stimulation at periosteal surface, such inhibitory action by androgen in osteoblasts at the endosteum is important for the maintenance of cortical width [220].

The specific effects of androgen on bone cells are mediated directly through androgen-receptors-signaling pathway (AR) (but there are also indirect contributions trough aromatization and estrogen receptors signalling). The regulation of AR expression in osteoblasts seems to be upregulation by androgens, which may result in an enhancement of androgen responsiveness at times when androgen levels are rising or elevated [216]. AR expression in osteoblasts can be upregulated by exposure to other steroid hormones, including glucocorticoids, estrogen or 1,25-dihydroxyvitamin D_3 [211]. ARs are also expressed in bone marrow stromal and mesenchymal precursor cells [230, 231], pluripotent cells that can differenciate into a variety of tissues including muscle, bone, and fat. Androgen may modulate precursor differentiation toward the osteoblast and/or myoblast lineage, while inhibiting differentiation toward the adipocite lineage [219]. These effects on stromal differentiation could underlie some of the consequences of androgen administration on body composition including increased muscle mass [232]. AR expression has also been detected in osteoclasts [233] and it has been shown that androgen treatment reduces bone resorption of isolated osteoclasts [200].

Anabolic steroids are used in the treatment of established osteoporosis in human patients, and it has been demonstrated that at least partly anabolic steroids increase the bone density by either stimulating bone formation or having anticatabolic effects. In men with idiopathic osteoporosis, the decrease of testosterone levels correlated with a decline in BMD, and testosterone replacement therapy increased BMD in the vertebrae [234]. In rat models of bone loss from hindlimb unweighting, administration of either testosterone or the synthetic androgen nandrolone blocked much of the immobilization-related decrease in BMD [235].

Nandrolone is an androgenic steroid that have been suggested to increase bone density by stimulating bone formation and is also known to have anti-catabolic effects [236] on bone. In the study described by Aithal et al. (2009) [204], nandrolone was evaluated for

their effect on mineralization in an animal model of osteopenia in growing rabbits. Most drugs used for osteoporosis have antiresorbing activity and thus prevent further loss of bone without increasing bone mass. ND has an acceptable side-effect profile, has shown its effectiveness in established osteoporosis with vertebral crush fractures and in corticosteroid osteoporosis [205-208].

In animals treated with Nandrolone, there was clear radiographic signs of bone mineralization and on days 45 and 60 after initiation of treatment. In rats and mice these agents are known to increase longitudinal and periosteal bone growth and bone mass, and also shown to decrease trabecular bone resorption [236]. ND has been shown to promote absorption of calcium from intestines and increase bone mineral content in women [237], stimulate endosteal bone formation in elderly dogs [236], and block bone loss by inhibition of bone resorption in ovariectomized rats with osteopenia [238]. Aithal et al. (2009) [239] also observed in their study that ND has shown some positive effect on mineralization of bones in growing rabbits with dietary induced osteopenia. It is likely that the drug may act by increasing the mineral deposition in the bone or by inhibiting mineral resorption; however, the exact mechanism of its action in growing animals with osteopenic bone conditions needs to be further investigated.

Geusens and Dequeker [240] demonstrated in a double-blind study with 34 patients with symptomatic osteoporosis that ND statistically significantly increased the BMC at the radius and reduced the endosteal bone loss at the metacarpals and the fracture rate in the second year observation period. Hassager et al. [241] showed that ND therapy could achieve an increase in BMC in postmenopausal women, but this was maintained only for as long as therapy was continued. ND is also effective in the prevention of corticosteroid-induced osteoporosis. This was evidenced by Adami et al. [242] who measured increased bone mass and decreased urinary excretion of hydroxyproline in patients on corticosteroid therapy receiving ND compared with control patients on corticosteroid therapy alone. Some studies have also shown that the effects of ND on bone were consistent with estradiol action and might be attributable to the increased serum estradiol [212].

The effects of ND treatment on bone mass and bone metabolism observed in Aerssens et al. [231] animal study are in line with the effects observed in humans [240, 243, 244], in beagle dogs [245], and in aged rats, but are not the same as the effects of estrogens. This finding is similar to results which Aerssens et al. (1993) found earlier in the bones

of growing children [246], where they demonstrated comparable effects of androgens and estrogens on trabecular bone density; however, androgens had a more pronounced effect on both cortical and trabecular BMC and bone sizes. This can be explained by a more pronounced stimulation of periosteal bone growth by androgens than by estrogens. Furthermore, a dissociative effect on biochemical markers for bone formation is observed in that study [231].

Aerssens et al. (1993) [247] concluded that ND has the unique ability to decrease bone turnover, characterized by decreased serum and bone osteocalcin levels, and to stimulate osteoblast activity, expressed by high serum alkaline phosphatase levels and increased IGF-I concentrations in the bone matrix, resulting in an increased cortical bone mass. The ND therapy associated increase in cortical bone mass and BMD is associated with improved mechanical properties, as expressed by bone stiffness and strength [247].

II. OBJECTIVES

The present study aimed a general objective: to investigate the effects of the amount and source of protein, resistance training and anabolic androgenic steroids administration on some structural bone parameters, weight and mineral content (such as calcium, phosphorus, magnesium and zinc) in the rat femur.

Under those specific experimental designed interventions, secondary objectives of this doctoral thesis are the following:

- to measure the digestive utilization of the above mentioned minerals.
- to determine the volumetric BMD in both trabecular and cortical bone by using the pQCT method.
- to check the renal regulation function to preserve the bone integrity by analysing calcium and citratum urinary excretions as well as the urine pH.
- to analyze the haemogram variations, plasmatic corticosterone and testosterone levels and, bone remodelling parameters.

II. OBJETIVOS

El objetivo general de esta Tesis Doctoral ha sido analizar los efectos de la cantidad y de la fuente proteica sobre parámetros óseos, el peso y el contenido de minerales totales y de calcio, fósforo, magnesio y zinc del fémur, y su relación con la utilización digestiva de esos minerales.

Los objetivos más específicos han sido:

- Determinar la densidad volumétrica del hueso trabecular y cortical, así como las propiedades estructurales del hueso por la técnica pQCT, que permite cuantificar los cambios que experimenta su geometría.
- Comprobar la función reguladora del riñón para preservar la integridad ósea, analizando la excreción urinaria de calcio, de citrato y el pH de la orina.
- Analizar los cambios a nivel del hemograma, niveles plasmáticos de corticosterona y testosterona, así como marcadores óseos de formación y resorción.

III. MATERIAL AND METHODS

1. Animals and experimental design

A total of 160 young male Wistar rats were allocated into sixteen groups derived of 4 main interventions: protein amount in the diet (HP vs. NP), protein source (whey vs. soy), training (RT vs. sedentary), and AAS (with AAS vs. without AAS administration) (Figure 1). Each specific intervention (i.e. HP diet, whey-protein diet, with RT and with AAS) was developed in groups of 10 rats. The experimental period lasted 3 months.

The animals, with an initial body weight of 150±8g, were housed from day 0 of the experiment in individual stainless steel metabolic cages designed for the separate collection and urine. The cages were located in a well-ventilated thermostatically controlled room (21±2ºC), with relative humidity ranging from 40 to 60%. A 12:12 reverse light-dark cycle (08.00–20.00 h) was implemented to allow exercise training during the day. Throughout the experimental period all rats had free access to double-distilled water and the animals consumed the four different diets (HP or NP, whey or soy protein) ad libitum. One week prior to the experimental period start, the rats were allowed to adapt to their respective diets and experimental conditions.

Body weight was measured weekly for all animals at the same time and the amount of food consumed by each rat was registered daily.

On week 11, a 12-hour urine sample from each animal was collected for biochemical analysis. During these 12 hours, located in the dark cycle, water was removed in order to avoid interferences with urine collection. Urine volumes were recorded and samples were transferred into graduated centrifuge tubes for the posterior pH, Ca, and citrate analysis.

At the end of the experimental period, the animals were anaesthetized with pentobarbital and sacrificed by exsanguination by means of cannulation of the abdominal aorta. Blood was collected (with heparin as anticoagulant) and centrifuged at 3000 rpm for 15 min to separate plasma that was frozen in liquid N and stored at -80ºC. Femurs were extracted, weighed, and immediately the left one was introduced in formalin for the morphological analysis and the right one was frozen in liquid N for the ashes analysis.

All experiments were undertaken according to Directional Guides Related to Animal Housing and Care (European Community Council, 1986) [248], and all procedures

were approved by the Animal Experimentation Ethics Committee of the University of Granada.

2. Experimental diets

Formulation of the experimental diets is presented in Table 1. All diets were formulated to cover the nutrient requirements of rats following the recommendations of the American Institute of Nutrition (AIN-93M) [249], with slight modifications. We have selected a 45% of protein level for the HP diet groups following previous studies in which HP diet was compared with NP diets in rats [46, 47, 218, 219], whereas a 10% protein content was chosen for the NP diet groups. Commercial whey or soy-protein isolates were used as the only protein source since this protein source is widely available and used by sportsmen. Inclusion of 45% protein level in the diet was done at the expense of complex carbohydrates (wheat starch).

Table 1. Formulation of the experimental diets

Nutritional Composition (g/100g DM)	Whey protein diet		Soy protein diet	
	Normal-protein	High-protein	Normal-protein	High-protein
Whey protein supplement	13.8	63.6	-	-
Soy protein supplement	-	-	13.1	57.4
Mineral mix (AIN-93M-MX)	3.5	3.5	3.5	3.5
Vitamin mix (AIN-93-VX)	1	1	1	1
Fat (olive oil)	4	4	4	4
Choline chloride	0.25	0.25	0.25	0.25
Cellulose	5	5	5	5
Starch	61.7	22.4	62.4	28.6
Methionine	0.5	-	0.5	-
Sucrose	10	-	10	-

2.1. Total nitrogen (N) content and total protein concentration

Prior to the diet preparation, total protein concentration of the commercial whey and soy hydrolyzates and its distribution among the protein or non-protein fractions was measured. Total N content of the commercial whey-protein hydrolyzates was 11.8±0.6g/100g of dry matter, which corresponds to a 73.8% of richness. Total N content of the commercial soy-protein hydrolyzate was 12.4±0.7g/100g of dry matter, which corresponds to a 77.5% of richness.

Total protein concentration of the experimental diets was also assayed, with values of 44.3±2.1 % and 10.4±0.6% for the HP and NP, respectively, whey-protein diet, and 44.1±2.2% and 9.8±0.4% for the HP and NP, respectively, soy-protein diet. These values are adequate for our experimental design.

3. Resistance training

3.1. Training protocol

The training protocol used in the present study has been described by Aparicio et al. (2011) [181]. The experimental group was trained following a resistance protocol in a motorised treadmill (Panlab TREADMILLS for five rats, LE 8710R) with weights in a bag tied with a cord to the tail (Fig. 2). This type of training was chosen in order to reproduce and mimic the type of exercise performed by people interested in gaining muscle mass and strength whose usually combine high-protein diets with AAS administration. Therefore, our training protocol follows the established principles for human RT, involving weights, repetitions and sets to maximize gains in muscle strength [250]. All the training process was designed and controlled by sport scientists in collaboration with experienced researches used to work with rats. We considered that a repetition was finished when the rat stopped running and the next repetition began when the rat started running again, as a consequence of the electric stimulus at the end of the treadmill. The repetitions usually lasted 2–4 s, and the number of repetitions per set ranged between eight and fourteen repetitions.

The training group exercised on alternate days (3–4 sessions/week). The animals ran at a constant speed of 40 cm/s during the whole experimental period (12 weeks) in their dark phase. Prior to exercise training, animals were adapted to the treadmill on a daily basis for 1 week, first three days without weight and the last four days with 20% of their body weights. The number of sessions performed each week on alternate days, the

number of sets per session and the time spent in each set as well as the load carried by the animals are shown in Table 2. From the first week of the experimental period until the completion of the study, the training weights (loads) were progressively increased and individually adjusted one time per week to the percentage of one repetition maximum (1 RM), defined as the maximum load that the rat could carry in the bag.

Animals in the control groups were managed identically to exercising animals, with the exception of exercise training. In order to avoid a possible confounding effect due to often handling in the training groups, control animals were handled weekly.

Table 2. Details of the resistance training programme

Weeks	Sessions/ week	Sets/ sessions	Set duration (min)	Rest between sets (min)	Load (% 1RM)
1	4	10	2	1	55
2	3	10	2	1	60
3	4	10	2	1	65
4	3	10	2	1.5	70
5	4	10	2	1.5	70
6	3	10	2.5	1.5	75
7	4	12	2.5	1.5	75
8	3	12	2	2	80
9	4	12	2.5	2	80
10	3	12	1.5	2	85
11	4	12	2	2.5	85
12	3	12	1	2.5	90

3.2. Repetition maximum test

The 1 RM test was conducted as follows: the rat was placed in a flat, horizontal and non-slippery surface with a specific loaded bag that was tied to its tail. The rat was acoustically stimulated and immediately reacted by moving forward. This procedure was repeated several times, increasing the load every time, until the load was so heavy that the rat could not move forward, yet actively stimulated. The load achieved at this point was considered the 1 RM and was weekly measured in all animals to adapt the %1 RM load during the training period.

4. Anabolic androgenic steroids administration

Following similar studies performed in rats, the animal received 10 mg/kg body weight of Nandrolone decanoate once a week by intramuscular injection in the gluteus (alternating the lateral side each week). This dose is comparable to the dose that has been reported as being frequently used by athletes (600 mg/week or approximately 8 mg/Kg/week) [222, 223]. We used a commercially available nandrolone decanoate solution of 50 mg/ml (Deca-Durabolin, Organon, Oss, Netherlands) that was diluted with saline solution 0,9% to appropriate concentrations for the lower doses to keep the volume of injection constant.

Control groups were injected with saline solution as placebo.

5. Chemical analyses

5.1. Moisture content

Moisture content of diets, protein hydrolyzates and bone were determined by drying to constant weight in a heater at 105 ± 1º C.

5.2. Total nitrogen (N) content

Total nitrogen content of the protein isolates, diets and tissues was determined according to Kjeldahl's method. Crude protein was calculated as N x 6.25.

5.3. Bone, diets and faeces ashes

Bone, diets and faeces ashes were prepared by calcination at 500ºC to a constant weight.

5.4. Mineral content in bone, diets and faeces

5.4.1. Calcium, Magnesium and Zinc determination

Calcium, magnesium and zinc content in bone, diets and faeces were determined by atomic absorption spectrophotometry using a Perkin Elmer Analyst 300 spectrophotometer (Perkin Elmer, Wellesley, MA, USA). Analytical results were validated by standard references certified reference material (CRM)-189 (wholemeal starch; Community Bureau of Reference, Geel, Belgium), CRM-383 (haricot beans; Community Bureau of Reference) and CRM-709 (pig feed; Community Bureau of Reference).

5.4.2. Phosphorus determination

Phosphorus content in bone, diets and faeces was determined by UV-Visible Spectrophotometer (BioMate 3S, Thermo Scientific).

5.5. Urinary parameters

5.5.1. Urine calcium content

Urine calcium content was determined by atomic absorption spectrophotometry (PerkinElmer, Wellesley, MA, USA).

5.5.2. Urinary Citrate

Urinary Citrate was measured using a commercial kit (Spinreact, S.A. Gerona, Spain).

5.5.3. Urinary pH

Urinary pH was analyzed using a bench pH-meter (Crison, Barcelona, Spain).

5.6. Plasmatic parameters

Plasma calcium, urea, total proteins, magnesium, phosphorous, iron and alkaline phosphatase, were measured using an autoanalyzer (Hitachi-Roche p800, F. Hoffmann-La Roche Ltd. Switzerland).

5.7. Hormone assay

5.7.1. Corticosterone: Plasma corticosterone concentrations were measured by radioimmunoassay (RIA) using a comercially available Corticosterone Rat/Mouse (DRG International Inc USA) I-125 Kit without modification. All samples were assayed in duplicate and in the same assay. The intra assay coeeficient of variation was 4,4% and the sensitivity was 7,7 ng/ml.

5.7.2. Testosterone: Plasma testosterone concentrations were measured by RIA using a comercially available TESTO-CTK (Dia Sorin,Italy) I-125 Kit without modification. All samples were assayed in duplicate and in the same assay. The intra assay coeeficient of variation was 5.1% and the sensitivity was 0.02 ng/ml.

6. Bone mineral density and structure measurements

Volumetric BMD of the left femur was measured by peripheral quantitative computed tomography (pQCT) using a XCT Research M+pQCT machine (Stratec Medizintechnik, Pforzheim, Germany) as described [251]. One slice (0.2mm thick) in the medial diaphysis of the femur, and 3 slices in the distal femoral metaphysis located 1.5, 2, and 2.5mm proximal to the articular surface of the knee joint were measured. Bone mineral density values of the distal femoral metaphysis were calculated as the mean over 3 slices. A voxel size of 0.070mm and a threshold of 600 mg/cm3 were used for calculation of cortical BMD. Trabecular BMD was calculated by using a threshold of 450 mg/cm3.

Measurements were made on two different areas of the femur: in the central part of the shaft, which has been chosen because it represents an area of predominantly cortical bone, while the measurements made in the metaphysis represent predominantly trabecular bone area.

7. Biological assesment

Changes in body weight (weight gain= final rat weight - initial rat weight/number of days) and food intake (final diet weight- initial diet weight/number of days) were measured.

Furthermore, total calcium, phosphorus, magnesium and zinc intakes, and faecal excretion of these minerals were analyzed.

7.1. Biological indices

The following indices and parameters were determined for each group according to the formulas given below:

Protein Efficiency Ratio (PER) (1), Food Transformation Index (FTI) (2), Apparent Digestibility Coefficient (ADC) (3) for calcium, phosphorus, magnesium and zinc:

PER= weight gained (g per rat per day)/protein intake (g per rat per day) (1)

FTI = Food Intake/ weight gain (2)

ADC = [(I-F)/I] X 100 (3)

where I is the intake, and F is the faecal excretion.

8. Statistical analysis

Results are presented as mean and standard error of the mean. The effects of the dietary protein amount and source, RT and AAS on the outcome variables were analyzed by four-ways ANOVA, with the four mentioned intervention groups as fixed factors, and the differents values as dependent variables in separate models. All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 16.0 for Windows; SPSS Inc., Chicago, IL), and the level of significance was set at 0.05.

IV. RESULTS

1. Chemical composition of the diets

The chemical composition of the diets used in the present study are showed in tables 1 and 3. All of them were adequate to cover the nutrient requirements of adult rats (National Research Council, 1995) with the exception of protein level of high-whey diet (HW) and high-soy diet (HS), due to the extra level added to these diets.

Calcium content in HW diet, and calcium, phosphorus, magnesium and zinc content of HS diet were higher due to the supplementation of these minerals originally present in the protein sources used for the present study.

2. Biological assessment

Daily food intake was significantly lower in HP groups, whey groups, resistance training and AAS injected groups ($P \leq 0.003$), and that lead to significantly lower weight gain in HP groups, resistance training groups and AAS injected groups ($P \leq 0.016$), see Table 4. Protein efficiency ratio (PER) was lower in rats fed HP diet ($P=0.000$). Food Transformation Index (FTI) were higher for rats followed a resistance training protocol and in rats treated with AAS ($P \leq 0.004$).

3. Digestive utilization of calcium

Calcium absorption (expressed as milligrams/day, table 5) was significant higher in groups fed soy protein diet, that also had a higher calcium intake when compared to the animals fed whey protein diet. The administration of AAS led to a significant reduction in calcium absorption compared to not AAS injected rats, which means a significant lower calcium intake and a significant higher faecal excretion. All these changes lead to significant lower ADC in AAS injected groups when compared to not AAS injected groups. No significant differences were found in digestive utilization parameters related to level of protein of diets neither for resistant training exercise, with the exception of a significant lower intake in rats followed a resistance training protocol.

4. Digestive utilization of magnesium

Magnesium absorption (expressed as milligrams/day, table 6) was higher in HP groups when compared to NP groups. Furthermore, HP groups had a significant higher magnesium intake and a higher ADC vs. NP groups.

Magnesium absorption, magnesium intake and magnesium faecal excretion were significant higher in rats fed soy protein diet when compared to the animals fed whey protein diet. All these changes led to a significant lower ADC in rats fed soy protein diet when compared to rats fed whey protein diet.

Absorbed magnesium and magnesium intake were lower in rats followed a resistance training protocol vs. sedentary rats, and in AAS injected groups vs. not AAS injected groups. All these changes led to a significant lower ADC in resistance training rats when compared to sedentary rats, and a lower ADC, but not significant, in rats AAS injected when compared to not AAS injected. No significant differences in faecal magnesium excretion attending to the level of protein of the diets, resistance training and AAS administration interventions were observed.

5. Final body weight, femur dry weight, femur ash, femur lenght and bone mineral content

The effects of the protein diet percentage, source of protein, resistance training and AAS-administration on final body weight, femur dry weight, femur ash, femur lenght and bone mineral content (calcium, magnesium, phosphorus and zinc) were measured (table 9).

5.1. Final body weight and femur dry weight

Final body weight was lower in resistance training groups when compared to sedentary groups and in AAS-injected groups when compared to not AAS-injected groups (respectively, $P<0.001$).

Furthermore, femur dry weight was lower in resistance training groups when compared to sedentary groups and femur ash weight was higher in HP groups vs. NP groups (respectively, $P<0.05$).

5.2. Femur length

Femur length was lower for the resistance training compared to the sedentary groups ($P<0.05$). No differences in femur length were found attending to the amount or source of protein and to AAS administration interventions.

5.3. Femur Calcium content

Femur Ca content in femur dry weight and in femur total ash were higher in the soy protein groups when compared to the whey protein groups and for the AAS injected when compared to not AAS injected groups (both, $P<0.001$). Whereas no differences were observed between HP vs. NP or exercise vs. sedentary groups.

5.4. Femur Magnesium content

Femur Mg content (mg/g dry weight, mg/g ash) was higher in training groups when compared to sedentary groups ($P=0.000$). Also it was higher (mgMg/g ash) in HP groups when compared to NP groups ($P<0.05$), and in the AAS injected groups compared to the non AAS injected groups (mgMg/g dry femur) ($P=0.002$). Femur Mg content (mg/g ash) was lower for the soy groups compared to whey groups ($P<0.05$).

5.5. Femur Phosphorus content

Femur P content (mg/g dry weight, mg/g ash) was lower for soy diet compared to whey diet, for training vs. sedentary groups and for AAS injected groups vs. not AAS injected groups ($P<0.001$). No differences were observed attending to the protein diet concentration.

5.6. Femur Zinc content

Femur Zn content (mg/g dry weight, mg/g ash) was higher for the HP groups compared to NP groups ($P=0.000$) and lower for soy protein vs. whey protein groups ($P=0.000$). No differences in Zn content were found attending to the resistance training and to AAS administration interventions.

6. Bone parameters in metaphysis and diaphysis of femur

The effects of the protein diet percentage, source of protein, resistance training and AAS-administration on different bone parameters in femur (metaphysis and diaphysis) were measured (table 10).

6.1. Methaphysis

Total BMD and cortical/subcortical BMD were higher in the exercise groups when compared to the sedentary groups (respectively, $P=0.006$ and $P=0.000$). No significant differences were observed between the normo protein (NP) and high protein (HP)

groups, between the vegetal protein (soy) groups when compared to the animal protein (whey) groups and between the AAS-injected compared to the not AAS-injected groups. Furthermore, no significat differences were observed in trabecular BMD attending to protein diet percentage, source of protein, resistance training or AAS-administration.

Total cross-sectional area and trabecular area were higher in the HP groups when compared to the NP groups (respectively, P=0.005 and P=0.006).
Total cross-sectional area and trabecular area had a significant decreased by the effect of resistance training (both P=0.000). Similar results were obtained with AAS treatment which also lead to a significant decrease of cortical/subcortical area ($P<0.05$).
No differences on total cross-sectional area in metaphysis were observed attending to the source of protein.

6.2. Diaphysis

No significant differences between groups were observed for the total BMD.

Total cross-sectional area in diaphysis was higher in the HP groups when compared to the NP groups (P=0.002). No significant differences between groups were observed attending to the source of protein, to the resistance training or to the AAS administration. Moreover, no significant differences were observed in cortical BMD attending to some intervention.
Whereas cortical area was higher in the HP groups when compared to the NP groups and in the soy groups when compared to whey groups (respectively, $P<0.05$), but it was lower in resistance training groups when compared to sedentary groups ($P<0.05$). No differences were observed attending to the AAS administration.

Cortical thickness was higher for soy groups compared to whey groups, but it was lower for training vs. sedentary groups (both $P<0.05$). Whereas no differences were observed between HP vs. NP groups or AAS-injected vs. not AAS-injected groups.

Periosteal perimeter and endocortical perimeter were higher in HP groups when compared to NP groups (respectively, $P<0.05$). However, no significant differences were observed attending to the source of protein, resistance training or AAS administration interventions.

7. Plasmatic parameters and hormone levels in serum

Plasmatic parameters and hormone levels in serum are showed in table 11.

Calcium levels in serum were higher in soy groups when compared to whey groups and in AAS-injected groups when compared to not AAS-injected (both, $P \leq 0.001$), and lower for resistance training groups when compared to sedentary groups ($P < 0.05$). No significant differences were found attending to the protein diet concentration.

Magnesium levels in serum were higher in AAS-injected groups when compared to not AAS-injected groups ($P < 0.05$). Whereas no differences were found attending to the concentration or source of protein and to resistance training interventions.

No significant differences were found in phosphorus serum levels.

Alkaline phosphatase levels in serum were higher in resistance training groups vs. sedentary groups and in AAS-injected groups vs. not AAS-injected groups (respectively, $P < 0.05$). No significant differences were observed attending to concentration or source of protein.

8. Urinary parameters

Urinary parameters are showed in table 12.

Calcium levels in urine were higher in HP groups vs. NP groups, and they were lower in soy groups vs. whey groups and in AAS-injected groups vs. not AAS-injected groups (all, $P = 0.000$). No significative differences were found in resistance training groups when compared to sedentary groups.

Citrate levels in urine were lower in HP groups when compared to NP groups and in resistance training groups when compared to sedentary groups, but they were higher in soy groups when compared to whey groups and in AAS-injected groups when compared to not AAS-injected groups (all, $P < 0.01$).

Urinary pH was lower in HP groups vs. NP groups, but it was higher in soy groups vs. whey groups (both, P=0.000). Whereas, no differences were observed attending to resistance training or AAS administration interventions.

V. DISCUSIÓN

En este estudio se investiga el efecto de cuatro intervenciones distintas sobre parámetros óseos, el peso y contenido de minerales totales y de calcio, fósforo, magnesio y zinc del fémur y su relación con la utilización digestiva de esos minerales. Por la importancia del calcio en relación con la fisiología del hueso, se ha determinado, además, la excreción urinaria de este mineral. Se incluyen también el hemograma, niveles plasmáticos de corticosterona y testosterona, así como marcadores óseos de formación y resorción.

La técnica pQCT permite determinar la densidad volumétrica del hueso trabecular y cortical, así como las propiedades estructurales del hueso, haciéndola una técnica ideal para cuantificar los cambios que experimenta su geometría.

Las intervenciones realizadas han sido:
1.- influencia de la cantidad de proteína de la dieta (normoproteica frente a hiperproteica)
2.- influencia del tipo de proteína de la dieta (proteína de origen animal-whey protein- frente a proteína de origen vegetal- soy protein-)
3.-Seguimiento de un protocolo de entrenamiento de fuerza (grupo sedentario frente a grupo con ejercicio)
4.- Administración de esteroides anabolizantes (grupo control frente a grupo inyectado diariamente con dosis de 10mg/kg de peso corporal de decanoato de nandrolona)

1. EFECTO DE LA CANTIDAD DE PROTEÍNA

La disminución de la ingesta que se observa en el grupo HP se asocia a una disminución significativa del incremento de peso expresado en gramos/rata/día, de forma similar a lo que ha sido descrito por otros autores [86, 181, 252-254], que relacionan la reducción del peso corporal con un efecto combinado de la menor ingesta de energía del grupo HP y al mayor gasto energético requerido para la digestión y metabolismo de los alimentos proteicos. Se ha descrito que el incremento de proteínas de la dieta da lugar, en ratas, a una elevación en plasma de los aminoácidos ramificados [255], que serían parcialmente responsables de una disminución del apetito [256], bien directamente debido al aumento en el cerebro de los aminoácidos libres, o bien indirectamente por bloquear la entrada al cerebro de aminoácidos neutros, incluidos el triptófano y la tirosina [257] que, como es sabido, son precursores en la síntesis de neurotransmisores implicados en la ingesta.

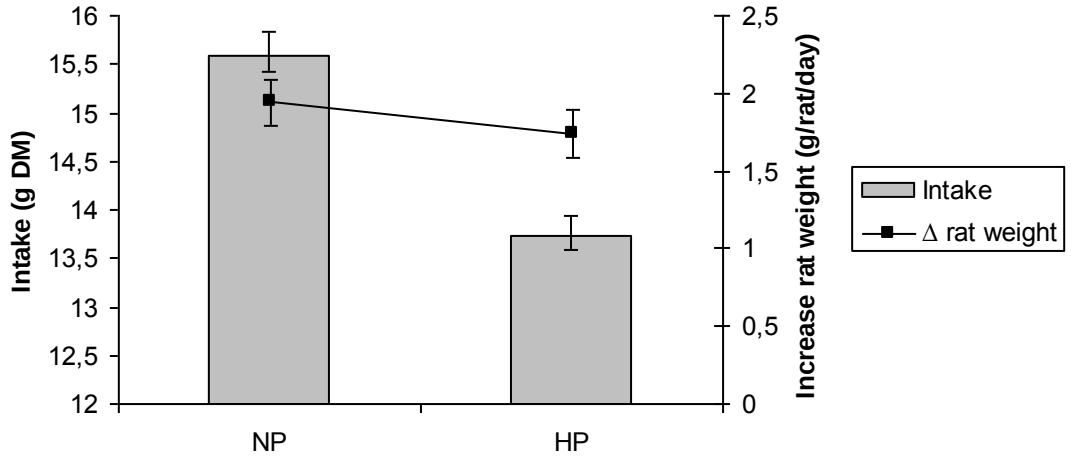

Figure 3. Effect of amount of protein on food intake and rat weight increase.
NP, normo-protein diet; HP, high-protein diet.

Asociado a esa menor ingesta de alimento y al menor incremento de peso hay una ingesta de proteína (g/d) muy superior en el grupo HP debido al alto porcentaje de proteína de esta dieta, y ello conduce a una disminución del PER, lo que indica un peor aprovechamiento de la utilización de proteínas con fines estructurales y un incremento del metabolismo de aminoácidos, lo que se refleja en el aumento significativo de niveles de urea en plasma [145, 246, 247] y menor nivel de proteínas plasmáticas (tabla 11). Las concentraciones plasmáticas de urea pueden incrementar cuando se consumen dietas HP [124, 229, 230]. En nuestro estudio hemos observado un incremento de casi el 50% en la concentración de urea plasmática en los grupos HP.

La mayor carga renal de urea implica una mayor tasa de filtración glomerular, siendo la diuresis mayor en un 48% en los grupos HP, este resultado fue observado por Aparicio et al. (2011) en nuestro laboratorio [258]. En nuestras condiciones experimentales, después de tres meses ingiriendo una dieta hiperproteica, se produjo una sobrecarga renal que se puso de manifiesto por un mayor peso del riñón y alteraciones morfológicas que han sido descritas por Aparicio et al. (2013) [259]. Paralelamente, se observa una disminución altamente significativa del pH urinario, hipocitraturia e hipercalciuria, factores todos ellos relacionados con el mayor riesgo de desarrollar litogénesis renal por una saturación de sales de calcio en orina [47, 232].

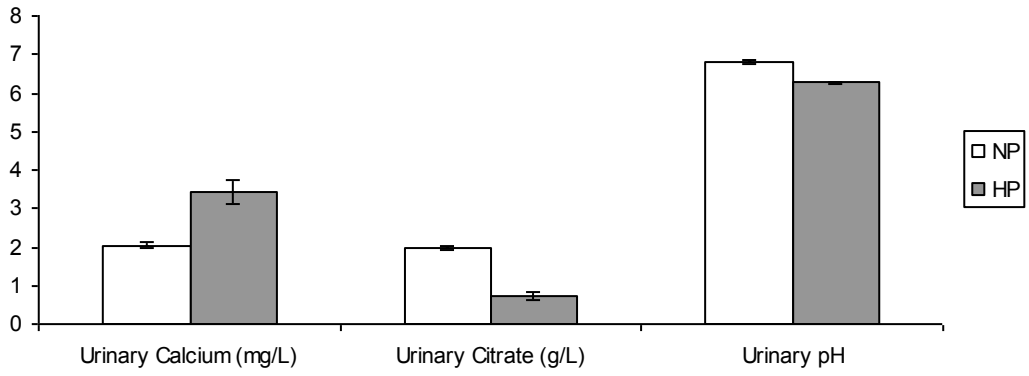

Figure 4. Effects of amount of protein on urinary parameters.
NP, normo-protein diet; HP, high-protein diet.

Uno de los nutrientes a los que prestamos mayor atención en nuestro estudio es el calcio, por su repercusión en la configuración del hueso. El metabolismo del calcio es complejo porque sus niveles plasmáticos dependen no sólo de la regulación renal, sino también de las variaciones en los niveles de absorción intestinal. En nuestro estudio hemos observado que la menor ingesta de alimento no afecta al nivel de calcio ingerido, como ocurre con la ingesta de proteína, porque la dieta HP tiene un contenido en calcio mayor que la NP y compensa la menor ingesta de alimento. Para niveles de calcio ingerido similares con ambas dietas se produce una disminución significativa en la absorción de calcio debido a la mayor excreción fecal, lo que se refleja en una disminución significativa del CDA. Existe controversia en cuanto al efecto de la cantidad de proteína sobre la absorción intestinal de calcio. La bibliografía nos indica [123] que no hay un efecto de la proteína de la dieta sobre la absorción de calcio determinada por técnicas isotópicas para dietas equilibradas, sin embargo, cuando se controla estrictamente la ingesta y se asocia a una disminución de proteína se produce un hiperparatiroidismo secundario que conduce a una disminución de la absorción de Ca.

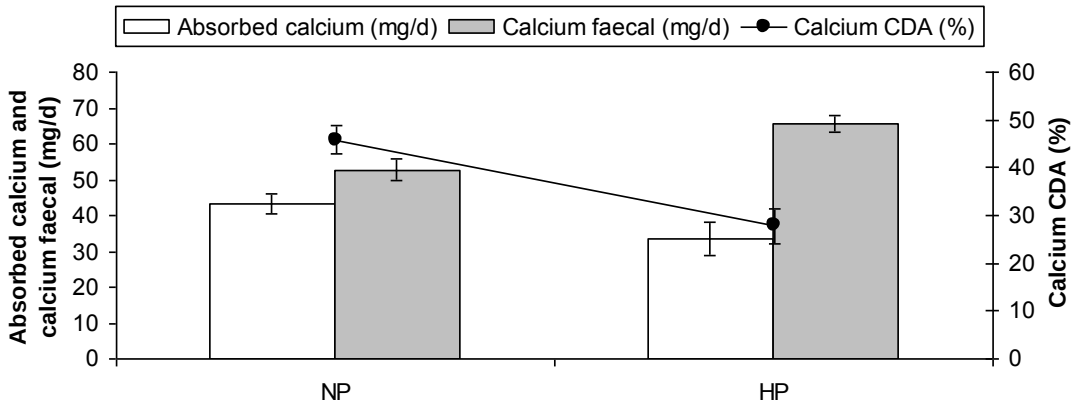

Figure 5. Effect of amount of protein on calcium digestive utilization.
NP, normo-protein diet; HP, high-protein diet.

A pesar de esto, en nuestro estudio, no se ve afectado el contenido de calcio en fémur (tabla 9) ni cuando se expresa por gramo de cenizas ni cuando se expresa por gramo de sustancia seca, ni la BMD de la metáfisis ni de la diáfisis, al tiempo que mejoran algunos parámetros relacionados con su geometría, concretamente, el área de la sección transversal de la metáfisis, así como su área trabecular; y en la diáfisis, el área de la sección transversal, el área cortical y los perímetros del periostio y del endostio. Los cambios a nivel de los perímetros se producen de una manera proporcional, ya que se mantiene el grosor cortical, e indican un aumento de la matriz orgánica del hueso (tabla 10). El mayor aporte de proteína de la dieta HP, además de proporcionar aminoácidos para la síntesis de la matriz orgánica, puede actuar de forma sinérgica con el calcio mejorando la retención y el metabolismo del hueso como han propuesto los autores Dawson-Hughes B, Harris SS, 2002; Conigrave AD et al., 2008; Cao JJ, Nielsen FH, 2010, y Kerstetter JE et al., 2011 [137-140]. Algunos estudios con isótopos también han demostrado una mayor retención y absorción de calcio en personas que consumen dietas HP, particularmente cuando el contenido en calcio de la dieta era limitado [140].

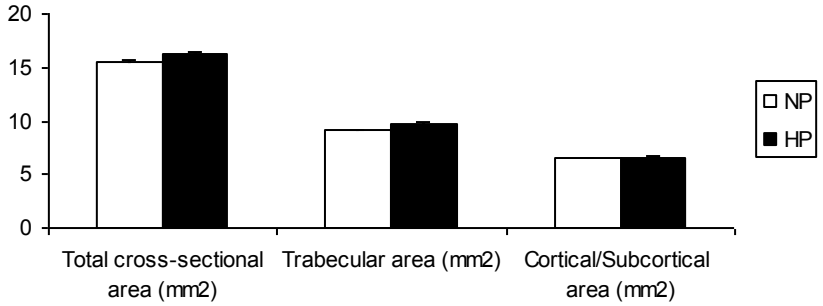

Figure 6. Effects of the amount of protein on some bone parameters of the metaphysis. NP, normo-protein diet; HP, high-protein diet.

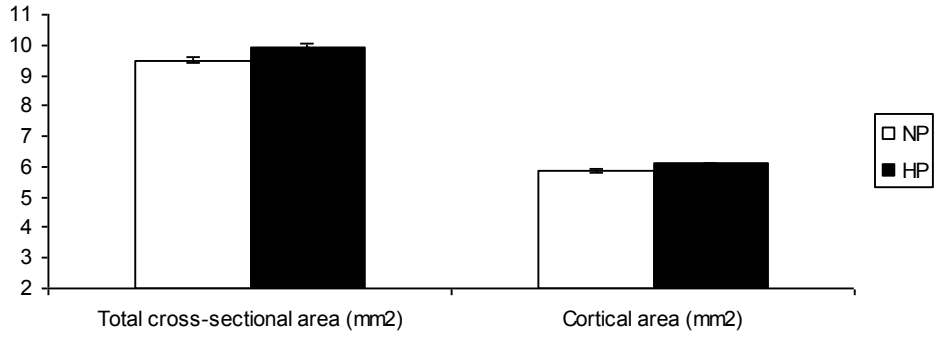

Figure 7. Effects of the amount of protein on some bone parameters of the diaphysis. NP, normo-protein diet; HP, high-protein diet.

Respecto al resto de minerales analizados (P, Mg y Zn), las dietas HP también proporcionan un aporte mineral muy elevado en relación a las NP. Esto conduce a una mayor absorción de estos minerales en valores absolutos, pero no en la absorción neta debido a los valores de excreción fecal obtenidos.

Los niveles plasmáticos de estos minerales no se modifican, pero en el hueso sí se observa un aumento en el contenido de Mg (/mg por gramo de cenizas de fémur) y en el de Zn (microgramos por gramo de fémur o por gramo de cenizas). Aunque tradicionalmente los estudios de minerales en el hueso se basan en el contenido de Ca y P, hay que destacar la importancia fisiológica de los cambios observados en cuanto al Mg y al Zn, ya que estos minerales actúan como cofactores de enzimas implicadas en la producción de energía (Mg y Zn). El Zn interviene en la actividad de más de 200 enzimas (Zn metalo-enzimas), en las que el Zn se localiza en el sitio activo, incluyendo

DNA polimerasa, RNA polimerasa, timidina quinasa y el sistema de defensa antioxidante [260].

2. EFECTO DE LA FUENTE PROTEICA

Hemos visto que la ingesta de los animales se modifica en función de la fuente proteica, consumen mayor cantidad de dieta experimental los animales alimentados con proteína de origen vegetal, coincidiendo con otros autores [260], y que los animales alcanzan un incremento de peso similar, con valores iguales de PER, que reflejan una adecuada utilización de la proteína ingerida en relación al incremento de peso en ambos grupos.

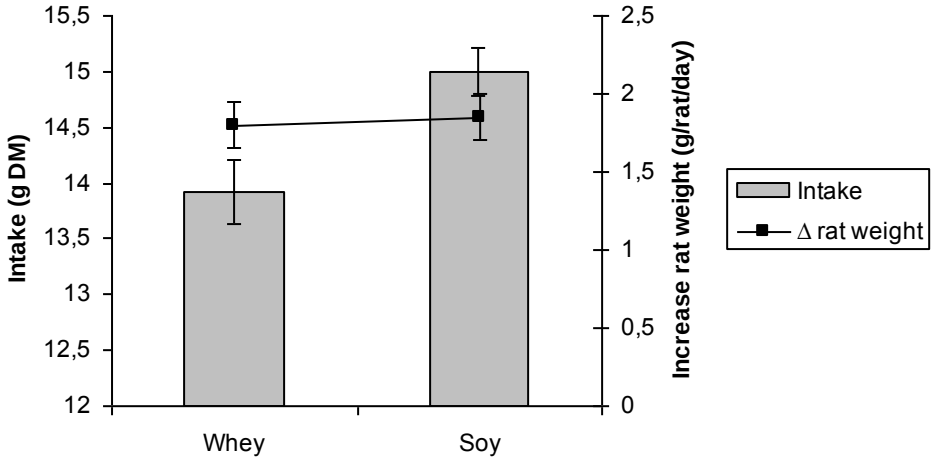

Figure 8. Effect of the source of protein on food intake and rat weight increase.

Zhou et al. (2011) [261] demostró que la proteína de lactosuero (whey) mostraba una disminución más acusada del apetito que la albúmina de huevo, la proteína de soja o la caseína [261-263]. La proteína whey es también más eficaz que la carne roja en reducir la ganancia de peso y en aumentar la sensibilidad a la insulina [253]. Estos estudios sugieren que las dietas HP de fuente proteica de origen animal (whey) pueden tener propiedades únicas en el mantenimiento saludable del peso corporal. La mayoría de los estudios sobre los efectos de la proteína whey en la disminución del apetito en humanos han sido a corto plazo, midiendo la ingesta de alimentos o bien, midiendo la saciedad varias horas después del consumo de una sola comida con proteína whey. En experimentos con animales, la dieta con proteína whey disminuía la ingesta de alimentos cuando se comparaba con la proteína de soja o con el gluten, durante un período de 7 días [262]. Sin embargo hay otros estudios a más largo plazo en los que la proteína whey no redujo significativamente la ingesta media diaria de alimentos en ratas

obesas durante 8 semanas de estudio [263]. La composición de aminoácidos de la proteína whey podría estar implicada en el mecanismo de la reducción de la ingesta de alimentos. La proteína whey, en comparación con las otras fuentes proteicas, es la proteína que contiene la concentración más alta de aminoácidos de cadena ramificada, concretamente del aminoácido L-leucina [264]. Este aminoácido puede pasar al cerebro desde la sangre más rápidamente que otros aminoácidos [265]. La leucina es importante en el control de la ingesta de alimentos y puede disminuir durante 24h la ingesta de alimentos en ratas. Esta acción del hipotálamo está relacionada con la regulación de la expresión del ARNm de un péptido que regula, a largo plazo, la ingesta de alimentos [266]. Por lo tanto, la dieta a base de proteína whey podría disminuir la ingesta de alimentos a través de su especial perfil de aminoácidos, especialmente la leucina, y alterando la síntesis de neuropéptidos a nivel hipotalámico, un lugar fundamental para el control de la ingesta de alimentos [261].

Los valores similares de urea plasmática, a diferencia de lo observado por efecto del aumento de la cantidad de proteína, reflejan una desaminación similar con las dos fuentes proteicas estudiadas. No obstante, se observan cambios muy significativos a nivel renal, apareciendo mayor excreción urinaria de calcio (mg/L), menor excreción urinaria de citratos y una acusada acidificación de la orina por efecto de la ingesta de proteína de lactosuero. En relación a la función renal es importante destacar su papel en la regulación del equilibrio ácido-base formando una orina más ácida o más básica, según las necesidades fisiológicas. En nuestras condiciones experimentales los cambios a nivel renal tras la ingesta de una dieta cuya fuente proteica es de origen animal pueden indicar una acidosis intracelular, como ha descrito Pak (2008) [108], debido a la carga ácida procedente de los aminoácidos azufrados. Se atribuye a esta acidosis intracelular la estimulación de la hipocitraturia, que se acompaña de hipercalciuria y que se relaciona fuertemente con la mayor excreción urinaria de protones [94].

Los cambios de la dieta animal sobre el riñón, además de ser un factor de riesgo de nefrolitiasis afectan también al hueso, en nuestras condiciones experimentales, provocando una disminución en el contenido de calcio, expresado en mg/g de fémur y en mg/g cenizas. La movilización de calcio del hueso puede ser el reflejo de una activación de la resorción ósea para poder mantener la homeostasis cálcica, de manera que el hueso ejerce una función tamponadora y reguladora de la calcemia a través de la liberación de calcio [88, 94, 267].

Como ya hemos comentado anteriormente, el catabolismo de las proteínas genera amonio y libera sulfato procedente de los aminoácidos. El citrato y el carbonato cálcico del hueso son movilizados para neutralizar la carga ácida que suponen esos aminoácidos, razón por la cual, la ingesta de proteína animal se asocia a mayor riesgo de osteoporosis que la proteína vegetal al promover la actividad osteoclástica e inhibir la actividad osteoblástica [100]. A pesar de la movilización de calcio del hueso, la calcemia presenta valores significativamente inferiores a la de los animales que ingieren la proteína vegetal, y en ello puede haber influido la menor ingesta de calcio, así como una menor absorción en valores absolutos. Sin embargo, no hemos encontrado movilización del resto de minerales analizados en fémur (Mg, P y Zn). Todos estos cambios no se correlacionan con alteraciones del BMD ni en diáfisis ni en metáfisis, pero sí se altera la geometría de la diáfisis por efecto de la proteína whey, ya que el fémur de estos animales presenta cantidades significativamente menores de área cortical y de grosor cortical. Proporcionalmente, han aumentado los valores de estos minerales en fémur cuando lo comparamos con el calcio.

A diferencia de lo que ocurre con la proteína whey, la bibliografía indica que el hidrolizado de soja contiene varios componentes que podrían beneficiar la salud del hueso, tales como isoflavonas y la baja composición en aminoácidos azufrados [268]. Según la bibliografía las isoflavonas contenidas en la soja estimulan la síntesis proteica y la expresión en osteoblastos [269], aunque nosotros no hemos encontrado diferencias significativas en los niveles de este marcador de formación ósea. Por otra parte, López-González et al. (2008) [143] indica que las poblaciones que consumen más proteína vegetal, tienden también a consumir más frutas, vegetales y el mineral potasio; estos factores ayudan a alcalinizar el medio y aportan efectos beneficiosos para el hueso.

3. EFECTO DEL ENTRENAMIENTO DE FUERZA

Las ratas que realizan el entrenamiento de fuerza ingieren menos cantidad de dieta que los animales sedentarios. Este hecho se correlaciona con un peso final del animal menor para los que practican el ejercicio de fuerza. Es bastante conocido que un entrenamiento de fuerza puede reducir el peso corporal [135, 270-272]. Esta reducción de peso se debe a 2 factores: 1) por una parte, por la disminución de la ingesta, en la que sin duda interviene la liberación de corticosterona promovida por el fuerte estrés del entrenamiento a largo plazo [273] 2) y, por otra, por los altos niveles plasmáticos de

corticosterona con su correspondiente efecto lipolítico sobre el tejido adiposo. La liberación de ácidos grasos libres aumenta el aporte de estos sustratos a los tejidos para obtener energía.

En general, para Ca, P, Mg y Zn la ingesta es significativamente menor por efecto del entrenamiento de fuerza, y está asociado a una menor absorción de estos minerales. Esto nos indica que los animales no han incrementado la absorción de nutrientes para compensar el menor aporte dietario. Además, los glucocorticoides disminuyen la absorción intestinal de calcio por la disminución de $1,25(OH)_2D_3$ renal.

Los efectos del ejercicio sobre el estado renal son controvertidos porque modifican distintos parámetros renales, algunos de los cuales mejoran la fisiología renal y otros que la perjudican. Se conoce que la inactividad o el sedentarismo contribuyen a la aparición de enfermedades crónicas del riñón [274]. El ejercicio mejora factores metabólicos y reduce la presión sanguínea, ayudando a conservar una adecuada funcionalidad en el riñón [243-245]. Bajo nuestras condiciones experimentales, la reducción del peso de los riñones tras un programa de ejercicio de fuerza durante tres meses en relación a los animales sedentarios, coincide con anteriores resultados de nuestro laboratorio [181], que habían puesto de manifiesto este efecto sobre el riñón en un grupo experimental más pequeño y cuya fuente proteica fue exclusivamente whey, pero además hemos encontrado un aumento de las proteínas plasmáticas lo que lleva aparejado un aumento de la presión oncótica del plasma y, por lo tanto, puede relacionarse con un efecto reductor de inflamación renal, tal y como encontraron Moinuddin I and Leehey DJ, 2008 y Poortmans JR y Ouchinsky M (2006) en humanos [275, 276].

Sin embargo, también hemos encontrado que los niveles de citrato urinario son menores en los animales que realizan el entrenamiento de fuerza, y esto es un efecto negativo en el riñón. El citrato urinario tiene un origen principalmente endógeno a través del ciclo de los ácidos tricarboxílicos y es bien conocido que el ejercicio intenso, como es el caso del entrenamiento de nuestros animales, disminuye el citrato urinario, tal y como ha sido descrito en la tesis doctoral de Aparicio VA (2012) [277]. Esto se puede explicar porque el entrenamiento de fuerza, probablemente, a través de un aumento de la corticosterona, muy pronunciado en nuestro estudio, produce cambios en el metabolismo celular y en la utilización de sustratos, obteniéndose la mayor parte de

energía necesaria a partir de los ácidos grasos. La citrato sintasa, enzima encargada de la síntesis del citrato, debe estar muy inhibida por dos razones: 1) por el aumento de cortisol que estimula la gluconeogénesis, induciendo la fosfanol piruvato sintasa, y por tanto, disminuyendo los niveles de oxalacetato, sustrato de la citrato sintasa; y 2) los niveles de ATP elevados tras la fuerte oxidación de ácidos grasos durante el ejercicio mantenido es un fuerte inhibidor de la citrato sintasa. Por todo ello los niveles d citrato plasmáticos estarán disminuidos y, en consecuencia, la excreción urinaria. Este resultado tiene un efecto potencialmente perjudicial para el riñón porque se ha demostrado que las concentraciones de elevadas de calcio y bajas de citrato en orina son los factores más importantes en la formación de piedras cálcicas, por el contrario cuando los niveles de citrato están elevados ejercen un inhibidor de la cristalización [278].

Sobre el hueso se han encontrado una serie de variaciones como consecuencia del entrenamiento. En primer lugar observamos que, en general, el fémur de estos animales tiene una longitud y un peso menor, con el mismo contenido de cenizas totales. La menor longitud del hueso se puede explicar probablemente debido a que se produce el cierre epifisario de forma prematura al iniciarse el protocolo de ejercicio en una etapa de desarrollo en la que aún no se ha completado la etapa de crecimiento, de acuerdo a lo descrito por Cossio-Bolaños (2012) [279], donde resalta que el crecimiento esquelético termina alrededor de los 120 días en ratas machos y hembras [280], llegando de esta forma a la adultez. En efecto, diversos autores han observado un retraso en el crecimiento del hueso largo en ratas sometidas a un entrenamiento prolongado, sin embargo, otros autores han encontrado resultados contradictorios, constatando aumentos en la talla con un ejercicio menos intenso. Parece probable que el efecto sobre el crecimiento está en función de la intensidad y de la duración del entrenamiento. Bailey (1978), en su capítulo de libro que se cita [281], indicó que Gelbeck (1892) estudió que cuando un hueso sometido a una fuerte tensión sacrifica su potencial de crecimiento para conservar su configuración y estabilidad [282].

Aunque las ratas después de tres meses de ejercicio tienen huesos más pequeños, su contenido mineral es similar al de las ratas sedentarias, lo que pone de manifiesto que, a pesar de los cambios a nivel digestivo y plasmático, el entrenamiento de fuerza ha evitado la movilización de calcio del hueso, manteniendo niveles iguales que las

controles, incluso se observa un incremento de BMD de la metáfisis, especialmente a nivel cortical/subcortical, pero no cambia la BMD trabecular, lo que sugiere que se hace más fuerte la superficie que el interior de la metáfisis, en la que además disminuye el área de la sección transversal, reflejándose este cambio en un menor área trabecular (sin afectarse el área cortical). Además, observamos que el riñón ejerce una función reguladora importante al evitar la pérdida de calcio por orina.

En este aumento de densidad puede haber contribuido el aumento del contenido en Mg tanto por g de fémur como por g de cenizas. El aumento de la fosfatasa alcalina, marcador de formación ósea, refleja esta mayor actividad osteoblástica [283, 284].

El fósforo no contribuye al aumento en la BMD de la metáfisis, puesto que sus valores son significativamente menores que en el fémur de las ratas sedentarias. La disponibilidad de fósforo (menor ingesta, menor CDA) ha sido significativamente menor, pero se mantienen los niveles plasmáticos del anión, además, parte del fósforo movilizado desde el hueso ha podido excretarse por orina ya que es conocido [285] que la PTH incrementa durante el entrenamiento de fuerza.

No se aprecian cambios relevantes en la diáfisis excepto en que observamos una disminución del área cortical y del grosor cortical. La disminución del área puede indicar que la cantidad de hueso formado puede no haber sido suficiente para mantener el grosor cortical adaptado apropiadamente al incremento de carga mecánica impuesta por el protocolo de ejercicio. Todo ello sugiere mayor riesgo de fracturas óseas.

Los principales cambios hemáticos observados (tabla 13) son un aumento significativo del hematocrito asociado a una disminución del hierro sérico. El ejercicio impone una mayor demanda de oxígeno para hacer frente a las necesidades energéticas de los tejidos e implica un mayor recambio de glóbulos rojos. En nuestras condiciones experimentales observamos una disminución en el contenido de hierro (microgramos/dL), un aumento en el hematocrito y un aumento en los niveles de bilirrubina. La disminución en los niveles de hierro sérico al mayor recambio de glóbulos rojos y a una menor ingesta con menor aporte general de nutrientes, entre ellos el hierro. En estas ratas entrenadas las proteínas plasmáticas aumentan, lo que conlleva un aumento de la presión osmótica y un aumento del volumen sanguíneo, pero no ocurre hemodilución (observamos niveles ligeramente mayores de RBC y de WBC) porque se postula que el entrenamiento a largo plazo ha producido un aumento gradual de la masa de hematíes y devuelve la relación entre la masa celular y el volumen total a niveles fisiológicos.

4. EFECTO DE LA ADMINISTRACIÓN DE ESTEROIDES ANABOLIZANTES

El decanoato de nandrolona (AAS inyectado en nuestro estudio) es un esteroide anabolizante cuyos efectos potenciales sobre los distintos tejidos están modulados a nivel celular, actuando, bien por una acción directa, o bien, por su transformación en estrógenos por un proceso de aromatización y transformándose en estradiol.

En primer lugar, hemos observado que el tratamiento con AAS disminuye significativamente la ingesta del alimento, y conduce a un menor incremento del peso de los animales. La disminución de la ingesta puede justificarse por el efecto inhibidor de la nandrolona sobre la expresión de adiponectina descrito por Alsio et al. (2009) [286]. A pesar del menor incremento de peso en los animales tratados hay un efecto de los AAS sobre la composición corporal, aumentando la masa muscular y descendiendo la masa grasa, probablemente la disminución de masa grasa es mayor que la ganancia de masa magra, de ahí la pérdida de peso. Este efecto anabólico de los AAS se pone de manifiesto por el aumento de la carcasa de los animales inyectados con AAS (resultados aún no publicados, pero incluidos en la tesis doctoral de Aparicio VA (2012) [277]. Hay que destacar el efecto significativo sobre el aumento del PER, que implica una mejor eficacia en el aprovechamiento de la proteína de la dieta, con una mejor relación entre la ganancia de peso en gramos por cada gramo de proteína ingerida. Nosotros hemos observado que el peso de los músculos cuadriceps y gastrocnemio es superior por efecto de los AAS, en parte, por el aumento en el contenido en agua (datos no publicados), lo que explicamos en parte por el efecto que tienen los AAS sobre la retención de líquidos, y en concreto por efecto del estradiol. En efecto, nuestros resultados (tabla 11) muestran que los AAS producen retención de Ca y Mg, que están significativamente más elevados en plasma, disminuyen la excreción urinaria en caso de calcio, y contribuyen de esta forma a la retención de agua. La mayor concentración de minerales plasmáticos favorece la mineralización ósea. Es llamativo que tienen un porcentaje significativamente superior de cenizas por gramo de fémur y los huesos son proporcionalmente de mayor peso (g/100g de peso de rata), con un contenido mayor en valores absolutos de Ca y Mg. La mayor concentración mineral no se refleja en un mayor BMD volumétrico, aunque sí se observan cambios en la geometría de la metáfisis con menor área de la sección transversal, con menor área trabecular y con menor área cortical/subcortical (tabla 10), produciéndose una alteración en las propiedades mecánicas del hueso. No observamos cambios en la diáfisis del fémur.

Respecto a la fosfatasa alcalina, nuestros resultados (tabla 11) concuerdan con la bibliografía, donde se describe que la administración de nandrolona aumenta significativamente los niveles de esta enzima, indicador de un activo remodelado de formación ósea.

El efecto de los AAS disminuyendo la excreción urinaria de calcio (mg/d) ha contribuido a la mejor mineralización del hueso. Este efecto regulador del riñón tiene especial importancia porque compensa, en parte, la menor ingesta y la menor absorción de Ca, Mg, P y Zn. Los AAS, además, aumentan citrato urinario y contribuyen a la alcalinización de la orina proporcionando un perfil renal menos propenso a la formación de cálculos.

Finalmente, en cuanto a los efectos observados sobre la ingesta y cambios ponderales (tabla 4), podríamos atribuir algunas alteraciones óseas al efecto del decanoato de nandrolona inyectado semanalmente a una dosis de 10 mg/kg de peso corporal durante 12 semanas, por el que los niveles plasmáticos del esteroide se han mantenido suficientemente elevados para conseguir un bloqueo de la producción de FSH y de LH al unirse a receptores del eje hipotálamo hipofisario, observándose una disminución significativa de los niveles plasmáticos de testosterona, y produciéndose una atrofia testicular (oligospermia), de acuerdo a lo que describieron Spitz et al. (1984) [287].

Por otra parte, hemos encontrado que el tratamiento con AAS reduce drásticamente los niveles de corticosterona, probablemente debido a la inhibición del eje hipotálamo hipofisario que se produce en tratamientos a largo plazo con nandrolona, como han descrito Schlussman et al. (2000) [288]. Además, según Alsio et al. (2009) [286], se produce una inhibición de las enzimas adrenales implicadas en la síntesis de corticosterona. Los cambios descritos en los niveles de testosterona y de corticosterona conducen a una relación corticosterona/testosterona más baja en las ratas inyectadas con AAS en relación a las no inyectadas, lo cual justifica los efectos anabólicos del esteroide (tabla 11).

VI. CONCLUSIONS

1.- High protein diet intake changed femur geometry by increasing the total cross-sectional and trabecular areas in bone metaphysis; while total cross sectional and cortical areas, and perisostium and endostium perimeters were increased in femur diaphysis. In this bone zone, large periostium and endostium perimeters were present along with an evident bone organic matrix development.

Although bone calcium content was maintained; this element excretion in urine was high, and urinary citrate and pH were low.

The significant high Mg and Zn levels detected in bone might have physiological implications due to their role as enzymatic cofactors.

2.- Diet incorporing proteic soy resulted in a higher food intake and absorbed calcium levels than whey protein diet. Consequently, high femur calcium and low P, Mg and Zn levels were related to some relevant bone changes such a larger cortical area and cortical thickness in femur diaphysis. Therefore, a mechanical resistance could be expected.

Moreover, soy protein intake improved the renal function by decreasing calcium urine excretion, by increasing citrate excretion and producing an alkaline urine.

3.- Resistance training retarded bone length development. High BMD in bone cortical/subcortical areas in metaphysis were found, that could be in relation to the training strengths in that specific area of the femur where high density and particular trabeculae disposition are present. Moreover, a decrease in total cross-sectional area was detected, generating a smaller trabecular area.

In bone diaphysis, a decrease in cortical area and cortical thickness were observed after resistance training. Those results showed a lower remodelling rate in the diaphysis than in the metaphysis, and therefore, resistance training might be inefficient to maintain a appropriate cortical thickness.

4.- Resistance training produced a decrease in food intake and in Ca, P, Mg y Zn absorption. Those findings might be also related to high corticosterone levels detected in stressed rats under resistance training protocol. Bone calcium was maintained in spite

of low absorption and plasmatic levels of calcium. It is important to emphasize the crucial kidney regulation function avoiding any calcium lost by urine excretion.

5.- Significant increase in haematocrit values associated to a decrease in serum iron were found under resistance training protocol. These results are related to low food intake and high levels of corticosterone that could be the cause of an overproduction of red blood cells.

6.- Anabolic steroids administration increased calcium and magnesium plasmatic levels, and decreased the calcium renal excretion. Thereby, producing bone mineralization and high weight bones. Also, higher ashes percentage and calcium and magnesium content were detected in femur. Although no changes in BMD were shown, geometric metaphysal changes such as a smaller total cross-sectional, trabecular and cortical/subcortical areas were found. Similar changes were not detected in femur diaphysis.

Finally, urinary citrate excretion was increased by anabolic steroids administration which benefits urine alkalinisation.

VI. CONCLUSIONES

1.- La ingesta de una dieta hiperproteica produce cambios en la geometría del fémur, aumentando el área de la sección transversal de la metáfisis, así como el área trabecular. En la diáfisis del fémur aumentan el área de la sección transversal, el área cortical y los perímetros del periostio y del endostio. Los cambios a nivel de los perímetros se producen de una manera proporcional, ya que se mantiene el grosor cortical, e indican un aumento de la matriz orgánica del hueso. El hueso mantiene su contenido en calcio a pesar de la alta excreción renal del catión, que se acompaña de una hipocitraturia acusada y de una acidificación de la orina. El aumento significativo de los niveles de Mg y Zn en el hueso puede tener una repercusión fisiológica por su función como cofactores de numerosas enzimas.

2.- La inclusión de soja como fuente proteica aumenta la cantidad de alimento ingerido y, la cantidad de calcio ingerido y absorbido producen cambios relevantes en el hueso frente a la proteína de lactosuero, que se refleja en un mayor contenido de calcio (por gramo de fémur y por gramo de ceniza de fémur), y menor contenido del resto de minerales estudiados (P, Mg y Zn), con mayor área cortical y mayor grosor cortical en la diáfisis, lo que sugiere una mayor resistencia mecánica.
Además, la ingesta de una dieta de proteína de soja mejora la fisiología renal, disminuyendo la excreción urinaria de calcio, aumentando la excreción de citrato y produciendo una orina más alcalina.

3.- Un entrenamiento de fuerza de alta intensidad produce un retraso en el crecimiento en longitud del hueso. Además, se produce un incremento de la BMD de la metáfisis, especialmente a nivel cortical/subcortical, probablemente debido a que las tensiones producidas por el ejercicio en esa zona del fémur posibilitan una disposición de trabéculas que aumenta la densidad, además disminuye el área de la sección transversal, reflejándose este cambio en una menor área trabecular. A nivel de la diáfisis, el entrenamiento de fuerza produce disminución en el área y en el grosor cortical que reflejan un remodelado menos dinámico que en la metáfisis y que indica que la cantidad de hueso formado puede no haber sido suficiente para mantener el grosor cortical

adaptado apropiadamente al incremento de carga mecánica impuesta por el protocolo de ejercicio.
El aumento de fosfatasa alcalina, marcador de formación ósea, refleja esta mayor actividad osteoblástica.

4.- La ingesta de alimento y de Ca, P, Mg y Zn es menor por efecto del entrenamiento de fuerza y esta asociada a una menor absorción de estos minerales, debido a los altos niveles de corticosterona circulante, reflejo del fuerte estrés al que están sometidos los animales por este protocolo desarrollado. Esta disminución en la absorción de calcio se refleja en unos valores plasmáticos menores. A pesar de los cambios a nivel digestivo y plasmático el entrenamiento ha evitado la movilización de calcio del hueso, el riñón ejerce una función reguladora importante al evitar la pérdida de calcio por orina, sin embargo, el nivel de citrato urinario excretado es menor.

5.- Como consecuencia del entrenamiento de fuerza los principales cambios hemáticos observados son un aumento significativo del hematocrito asociado a una disminución del hierro sérico, debido a una menor ingesta con menor aporte general de nutrientes, entre ellos el hierro, y a los altos niveles de corticosterona que producen un mayor recambio de células sanguíneas.

6.- La administración de esteroides anabolizantes aumenta los niveles de calcio y magnesio en plasma y disminuyen la excreción renal de calcio, estos cambios favorecen la mineralización ósea, conduciendo a huesos de peso mayor, mayor porcentaje de cenizas y con un contenido mayor de Ca y Mg por gramo de fémur y por gramo de cenizas. La mayor concentración mineral no se refleja en un mayor BMD volumétrico, aunque sí afecta a la geometría de la metáfisis con un menor área de la sección transversal, un menor área trabecular y menor área cortical/subcortical, sin producirse cambios a nivel de diáfisis.
Los AAS, además, aumentan el citrato urinario y contribuyen a la alcalinización de la orina.

VII. REFERENCES

1. Smith, R., *Bone mineral,* in *Human Nutrition and Dietetics,* J.S. Garrow, Editor. 2000. p. 165-175.
2. Marcus R, F.D., Kelsey J, *Osteoporosis.* Academic Press, New York, 1996.
3. Fraser, Lancet, 1995(345): p. 104-107.
4. Suda, T., et al., *Vitamin D and bone.*
5. Carlsson, A., *Tracer Experiments on the Effect of Vitamin D on the Skeletal Metabolism of Calcium and Phosphorus.*
6. Udagawa, N., et al., *Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells.* Proc Natl Acad Sci U S A, 1990. **87**(18): p. 7260-4.
7. Takahashi, N., et al., *OSTEOBLASTIC CELLS ARE INVOLVED IN OSTEOCLAST FORMATION.* Endocrinology, 1988. **123**(5): p. 2600-2602.
8. Brown, E.M., et al., *Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid.* Nature, 1993. **366**(6455): p. 575-80.
9. Brown, E.M., *Mechanisms underlying the regulation of parathyroid hormone secretion in vivo and in vitro.* Curr Opin Nephrol Hypertens, 1993. **2**(4): p. 541-51.
10. Grey, A., et al., *Parallel phosphatidylinositol-3 kinase and p42/44 mitogen-activated protein kinase signaling pathways subserve the mitogenic and antiapoptotic actions of insulin-like growth factor I in osteoblastic cells.* Endocrinology, 2003. **144**(11): p. 4886-93.
11. Hock, J.M., E. Canalis, and M. Centrella, *Transforming growth factor-beta stimulates bone matrix apposition and bone cell replication in cultured fetal rat calvariae.* Endocrinology, 1990. **126**(1): p. 421-6.
12. Qin, L., et al., *Parathyroid hormone uses multiple mechanisms to arrest the cell cycle progression of osteoblastic cells from G1 to S phase.* J Biol Chem, 2005. **280**(4): p. 3104-11.
13. Dobnig, H. and R.T. Turner, *Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells.* Endocrinology, 1995. **136**(8): p. 3632-8.
14. Rosen, C., Niu, T., *Insulin-loke growth factors and the IGF binding proteins: Implications for Bone Biology,* in *Principles of Bone Biology,* J.P. bilezikian, Raisz, L. G., Martin, T. J., Editor. 2008. p. 1069-1094.
15. Pilbeam CC, C.S., Blackwell K and Raisz LG, *Prostaglandins and bone metabolism,* in *Principles of Bone Biology,* J.P. Bilezikian, Raisz, L. G., Martin, T. J., Editor. 2008. p. 1235-1271.
16. Akhter, M.P., et al., *Bone biomechanical properties in prostaglandin EP1 and EP2 knockout mice.* Bone, 2001. **29**(2): p. 121-5.
17. Brochhausen, C., et al., *Cyclooxygenases and prostaglandin E2 receptors in growth plate chondrocytes in vitro and in situ--prostaglandin E2 dependent proliferation of growth plate chondrocytes.* Arthritis Res Ther, 2006. **8**(3): p. R78.
18. Bevington A, K.G.J., Russell R G G, *Acquired disorders of phosphate metabolism,* in *The metabolic and molecular basis of acquired disease,* L.B. Cohen R D, Alberti K G M M, Denman A M, Editor. 1990, Bailliére Tindall: London. p. 1097.

19. Drezner, M.K., *Phosphorus Homeostasis and Related Disorders*, in *Principles of Bone Biology*, J.P. Bilezikian, Raisz, L. G., Martin, T. J., Editor. 2008, Elsevier. p. 465-486.
20. Rude R K, O.S.B., *Disorders of magnesium metabolism*, in *The metabolic and molecular basis of acquired disease*, L.B. Cohen R D, Alberti K G M M, Denman A M, Editor. 1990, Bailliére Tindall: London. p. 1124-1148.
21. Rude, R., *Magnesium Homeostasis*, in *Principles of Bone Biology*, J.P. bilezikian, Raisz, L. G., Martin, T. J., Editor. 2008, Elsevier. p. 487-513.
22. Sherwood, L.M., I. Herrman, and C.A. Bassett, *Parathyroid hormone secretion in vitro: regulation by calcium and magnesium ions.* Nature, 1970. **225**(5237): p. 1056-8.
23. Cholst, I.N., et al., *The influence of hypermagnesemia on serum calcium and parathyroid hormone levels in human subjects.* N Engl J Med, 1984. **310**(19): p. 1221-5.
24. Ferment, O., P.E. Garnier, and Y. Touitou, *Comparison of the feedback effect of magnesium and calcium on parathyroid hormone secretion in man.* J Endocrinol, 1987. **113**(1): p. 117-22.
25. Toffaletti, J., D.L. Cooper, and B. Lobaugh, *The response of parathyroid hormone to specific changes in either ionized calcium, ionized magnesium, or protein-bound calcium in humans.* Metabolism, 1991. **40**(8): p. 814-8.
26. Shils, R.R.a.M., *Magnesium*, in *Modern Nutrition in Health and Disease*, S. ME, Editor. 2006, Lippincott Williams and Wilkins: Philadelphia.
27. Miki, H., P.B. Maercklein, and L.A. Fitzpatrick, *Effect of magnesium on parathyroid cells: evidence for two sensing receptors or two intracellular pathways?* Am J Physiol, 1997. **272**(1 Pt 1): p. E1-6.
28. Burnell, J.M., et al., *Effects of dietary alteration of bicarbonate and magnesium on rat bone.* Am J Physiol, 1986. **250**(2 Pt 2): p. F302-7.
29. Boskey, A.L., et al., *Effect of short-term hypomagnesemia on the chemical and mechanical properties of rat bone.* J Orthop Res, 1992. **10**(6): p. 774-83.
30. M. Kaji, Y.N., *Growth and Minerals: Zinc.* Growth, Genetics & Hormones, 2006. **22**(1): p. 1-7.
31. Nishi, Y., *Zinc and growth.* J Am Coll Nutr, 1996. **15**(4): p. 340-4.
32. Yamaguchi, M., *Role of zinc in bone formation and bone resorption.* Trace Elem Exper Med., 1998(11): p. 119-135.
33. Hall, S.L., H.P. Dimai, and J.R. Farley, *Effects of zinc on human skeletal alkaline phosphatase activity in vitro.* Calcif Tissue Int, 1999. **64**(2): p. 163-72.
34. Yamaguchi, M. and K. Inamoto, *Differential effects of calcium-regulating hormones on bone metabolism in weanling rats orally administered zinc sulfate.* Metabolism, 1986. **35**(11): p. 1044-7.
35. McDonnell, D.P., et al., *Molecular cloning of complementary DNA encoding the avian receptor for vitamin D.* Science, 1987. **235**(4793): p. 1214-7.
36. Low, M.G. and A.R. Saltiel, *Structural and functional roles of glycosyl-phosphatidylinositol in membranes.* Science, 1988. **239**(4837): p. 268-75.
37. Crofton, P.M., *Biochemistry of alkaline phosphatase isoenzymes.* Crit Rev Clin Lab Sci, 1982. **16**(3): p. 161-94.
38. Calvo, M.S., D.R. Eyre, and C.M. Gundberg, *Molecular basis and clinical application of biological markers of bone turnover.* Endocr Rev, 1996. **17**(4): p. 333-68.

39. Bowles, S.A., et al., *Serum osteocalcin, total and bone-specific alkaline phosphatase following isolated tibial shaft fracture.* Ann Clin Biochem, 1996. **33 (Pt 3)**: p. 196-200.
40. Woitge, H.W., M.J. Seibel, and R. Ziegler, *Comparison of total and bone-specific alkaline phosphatase in patients with nonskeletal disorder or metabolic bone diseases.* Clin Chem, 1996. **42**(11): p. 1796-804.
41. Gundberg, C.M., et al., *Osteocalcin in human serum: a circadian rhythm.* J Clin Endocrinol Metab, 1985. **60**(4): p. 736-9.
42. Obrant, K.J., et al., *Serum bone-gla protein after fracture.* Clin Orthop Relat Res, 1990(258): p. 300-3.
43. Olsen, B.R., et al., *Purification and characterization of a peptide from the carboxy-terminal region of chick tendon procollagen type I.* Biochemistry, 1977. **16**(13): p. 3030-6.
44. Smedsrod, B., et al., *Circulating C-terminal propeptide of type I procollagen is cleared mainly via the mannose receptor in liver endothelial cells.* Biochem J, 1990. **271**(2): p. 345-50.
45. Kivirikko KI, M., R., *Biosynthesis of the collagens*, in *Extracellular Matrix Biochemistry*, K.A.a.R.A. Piez, Editor. 1980, Elsevier: New York. p. 83-118.
46. Eriksen, E.F., et al., *Serum markers of type I collagen formation and degradation in metabolic bone disease: correlation with bone histomorphometry.* J Bone Miner Res, 1993. **8**(2): p. 127-32.
47. Hassager, C., et al., *The carboxy-terminal propeptide of type I procollagen in serum as a marker of bone formation: the effect of nandrolone decanoate and female sex hormones.* Metabolism, 1991. **40**(2): p. 205-8.
48. Hassager, C., et al., *Diurnal variation in serum markers of type I collagen synthesis and degradation in healthy premenopausal women.* J Bone Miner Res, 1992. **7**(11): p. 1307-11.
49. Saggese, G., et al., *Twenty-four-hour osteocalcin, carboxyterminal propeptide of type I procollagen, and aminoterminal propeptide of type III procollagen rhythms in normal and growth-retarded children.* Pediatr Res, 1994. **35**(4 Pt 1): p. 409-15.
50. Ingle, B.M., et al., *Changes in bone mass and bone turnover following ankle fracture.* Osteoporos Int, 1999. **10**(5): p. 408-15.
51. Ingle, B.M., et al., *Changes in bone mass and bone turnover following distal forearm fracture.* Osteoporos Int, 1999. **10**(5): p. 399-407.
52. Prockop, D.J., *ISOTOPIC STUDIES ON COLLAGEN DEGRADATION AND THE URINE EXCRETION OF HYDROXYPROLINE.* J Clin Invest, 1964. **43**: p. 453-60.
53. Eyre, D.R., T.J. Koob, and K.P. Van Ness, *Quantitation of hydroxypyridinium crosslinks in collagen by high-performance liquid chromatography.* Anal Biochem, 1984. **137**(2): p. 380-8.
54. Eyre, D.R., I.R. Dickson, and K. Van Ness, *Collagen cross-linking in human bone and articular cartilage. Age-related changes in the content of mature hydroxypyridinium residues.* Biochem J, 1988. **252**(2): p. 495-500.
55. Seibel, M.J., S.P. Robins, and J.P. Bilezikian, *Urinary pyridinium crosslinks of collagen: specific markers of bone resorption in metabolic bone disease.* Trends Endocrinol Metab, 1992. **3**(7): p. 263-70.
56. Robins, S.P., *Fibrillogenesis and maduration of collagens*, in *Dynamics of bone and cartilage metabolism*, R.S. Seibel MJ, Bilezikian JP., Editor. 2006, Academic Press. p. 41-53.

57. Eastell, R., et al., *Biochemical markers of bone resorption compared with estimates of bone resorption from radiotracer kinetic studies in osteoporosis.* J Bone Miner Res, 1997. **12**(1): p. 59-65.
58. Garnero, P., et al., *The type I collagen fragments ICTP and CTX reveal distinct enzymatic pathways of bone collagen degradation.* J Bone Miner Res, 2003. **18**(5): p. 859-67.
59. Risteli, J., Risteli, L., *Products of bone collagen metabolism*, in *Dynamics of Bone and Cartilage Metabolism*, R.S.P. Seibel M. J., Bilezikian J.P., Editor. 1999, Academics Press: San Diego. p. 275-288.
60. Magnusson, P., et al., *Effect of chronic renal failure on bone turnover and bone alkaline phosphatase isoforms.* Kidney Int, 2001. **60**(1): p. 257-65.
61. Fall, P.M., et al., *Comparison of serum and urine assays for biochemical markers of bone resorption in postmenopausal women with and without hormone replacement therapy and in men.* Osteoporos Int, 2000. **11**(6): p. 481-5.
62. Okuno, S., et al., *Serum levels of C-terminal telopeptide of type I collagen: a useful new marker of cortical bone loss in hemodialysis patients.* Osteoporos Int, 2005. **16**(5): p. 501-9.
63. Seibel, M.J., *Biochemical markers of bone turnover: part I: biochemistry and variability.* Clin Biochem Rev, 2005. **26**(4): p. 97-122.
64. Guanabens, N., et al., *Collagen-related markers of bone turnover reflect the severity of liver fibrosis in patients with primary biliary cirrhosis.* J Bone Miner Res, 1998. **13**(4): p. 731-8.
65. Qvist, P., et al., *Circadian variation in the serum concentration of C-terminal telopeptide of type I collagen (serum CTx): effects of gender, age, menopausal status, posture, daylight, serum cortisol, and fasting.* Bone, 2002. **31**(1): p. 57-61.
66. Hamano, T., et al., *Serum NTX is a practical marker for assessing antiresorptive therapy for glucocorticoid treated patients with chronic kidney disease.* Bone, 2006. **39**(5): p. 1067-72.
67. Gertz, B.J., et al., *Application of a new serum assay for type I collagen cross-linked N-telopeptides: assessment of diurnal changes in bone turnover with and without alendronate treatment.* Calcif Tissue Int, 1998. **63**(2): p. 102-6.
68. Greenspan, S.L., et al., *Diurnal variation of bone mineral turnover in elderly men and women.* Calcif Tissue Int, 1997. **60**(5): p. 419-23.
69. Segrest, J.P. and L.W. Cunningham, *Variations in human urinary O-hydroxylysyl glycoside levels and their relationship to collagen metabolism.* J Clin Invest, 1970. **49**(8): p. 1497-509.
70. Krane, S.M., et al., *Urinary excretion of hydroxylysine and its glycosides as an index of collagen degradation.* J Clin Invest, 1977. **59**(5): p. 819-27.
71. Minkin, C., *Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function.* Calcif Tissue Int, 1982. **34**(3): p. 285-90.
72. Yam, L.T., *Clinical significance of the human acid phosphatases: a review.* Am J Med, 1974. **56**(5): p. 604-16.
73. Hannon, R.A., et al., *Clinical performance of immunoreactive tartrate-resistant acid phosphatase isoform 5b as a marker of bone resorption.* Bone, 2004. **34**(1): p. 187-94.
74. Stepan, J.J., et al., *Serum osteocalcin, bone alkaline phosphatase isoenzyme and plasma tartrate resistant acid phosphatase in patients on chronic maintenance hemodialysis.* Bone Miner, 1987. **3**(2): p. 177-83.

75. Bellahcene, A., et al., *Bone sialoprotein mediates human endothelial cell attachment and migration and promotes angiogenesis.* Circ Res, 2000. **86**(8): p. 885-91.
76. Seibel, M.J., et al., *Serum immunoreactive bone sialoprotein as a new marker of bone turnover in metabolic and malignant bone disease.* J Clin Endocrinol Metab, 1996. **81**(9): p. 3289-94.
77. Shaarawy, M. and M. Hasan, *Serum bone sialoprotein: a marker of bone resorption in postmenopausal osteoporosis.* Scand J Clin Lab Invest, 2001. **61**(7): p. 513-21.
78. Garnero, P., et al., *The collagenolytic activity of cathepsin K is unique among mammalian proteinases.* J Biol Chem, 1998. **273**(48): p. 32347-52.
79. Kafienah, W., et al., *Human cathepsin K cleaves native type I and II collagens at the N-terminal end of the triple helix.* Biochem J, 1998. **331 (Pt 3)**: p. 727-32.
80. Li, Z., et al., *Regulation of collagenase activities of human cathepsins by glycosaminoglycans.* J Biol Chem, 2004. **279**(7): p. 5470-9.
81. Goto, T., T. Yamaza, and T. Tanaka, *Cathepsins in the osteoclast.* J Electron Microsc (Tokyo), 2003. **52**(6): p. 551-8.
82. Sellmeyer, D.E., et al., *A high ratio of dietary animal to vegetable protein increases the rate of bone loss and the risk of fracture in postmenopausal women. Study of Osteoporotic Fractures Research Group.* Am J Clin Nutr, 2001. **73**(1): p. 118-22.
83. Kerstetter, J.E., et al., *Changes in bone turnover in young women consuming different levels of dietary protein.* J Clin Endocrinol Metab, 1999. **84**(3): p. 1052-5.
84. Talbott, S.M., et al., *Energy restriction reduces bone density and biomechanical properties in aged female rats.* J Nutr, 2001. **131**(9): p. 2382-7.
85. Weiss, R.E., et al., *Influence of high protein diets on cartilage and bone formation in rats.* J Nutr, 1981. **111**(5): p. 804-16.
86. Lacroix, M., et al., *A long-term high-protein diet markedly reduces adipose tissue without major side effects in Wistar male rats.* Am J Physiol Regul Integr Comp Physiol, 2004. **287**(4): p. R934-42.
87. Amanzadeh, J., et al., *Effect of high protein diet on stone-forming propensity and bone loss in rats.* Kidney Int, 2003. **64**(6): p. 2142-9.
88. Mardon, J., et al., *Long-term intake of a high-protein diet with or without potassium citrate modulates acid-base metabolism, but not bone status, in male rats.* J Nutr, 2008. **138**(4): p. 718-24.
89. Matsuo, T., et al., *Effects of voluntary resistance exercise and high-protein snack on bone mass, composition, and strength in rats given glucocorticoid injections.* Biosci Biotechnol Biochem, 2003. **67**(12): p. 2518-23.
90. Alexy, U., et al., *Long-term protein intake and dietary potential renal acid load are associated with bone modeling and remodeling at the proximal radius in healthy children.* Am J Clin Nutr, 2005. **82**(5): p. 1107-14.
91. Promislow, J.H., et al., *Protein consumption and bone mineral density in the elderly : the Rancho Bernardo Study.* Am J Epidemiol, 2002. **155**(7): p. 636-44.
92. Wachman, A. and D.S. Bernstein, *Diet and osteoporosis.* Lancet, 1968. **1**(7549): p. 958-9.
93. Massey, L.K., *Dietary animal and plant protein and human bone health: a whole foods approach.* J Nutr, 2003. **133**(3): p. 862S-865S.

94. Tylavsky, F.A., L.A. Spence, and L. Harkness, *The importance of calcium, potassium, and acid-base homeostasis in bone health and osteoporosis prevention.* J Nutr, 2008. **138**(1): p. 164S-165S.
95. Green, J. and C.R. Kleeman, *Role of bone in regulation of systemic acid-base balance.* Kidney Int, 1991. **39**(1): p. 9-26.
96. Remer, T., *Influence of diet on acid-base balance.* Semin Dial, 2000. **13**(4): p. 221-6.
97. Frassetto, L., et al., *Diet, evolution and aging--the pathophysiologic effects of the post-agricultural inversion of the potassium-to-sodium and base-to-chloride ratios in the human diet.* Eur J Nutr, 2001. **40**(5): p. 200-13.
98. New, S.A., *Intake of fruit and vegetables: implications for bone health.* Proc Nutr Soc, 2003. **62**(4): p. 889-99.
99. Krieger, N.S., N.E. Sessler, and D.A. Bushinsky, *Acidosis inhibits osteoblastic and stimulates osteoclastic activity in vitro.* Am J Physiol, 1992. **262**(3 Pt 2): p. F442-8.
100. Alpern, R.J. and K. Sakhaee, *The clinical spectrum of chronic metabolic acidosis: homeostatic mechanisms produce significant morbidity.* Am J Kidney Dis, 1997. **29**(2): p. 291-302.
101. Remer, T., *Influence of nutrition on acid-base balance--metabolic aspects.* Eur J Nutr, 2001. **40**(5): p. 214-20.
102. Jesudason, D. and P. Clifton, *The interaction between dietary protein and bone health.* J Bone Miner Metab, 2011. **29**(1): p. 1-14.
103. Bonjour, J.P., *Dietary protein: an essential nutrient for bone health.* J Am Coll Nutr, 2005. **24**(6 Suppl): p. 526S-36S.
104. Kannus, P., et al., *Epidemiology of hip fractures.* Bone, 1996. **18**(1 Suppl): p. 57S-63S.
105. Weisinger, J.R., et al., *Possible role of cytokines on the bone mineral loss in idiopathic hypercalciuria.* Kidney Int, 1996. **49**(1): p. 244-50.
106. Allen, L.H., E.A. Oddoye, and S. Margen, *Protein-induced hypercalciuria: a longer term study.* Am J Clin Nutr, 1979. **32**(4): p. 741-9.
107. Hunt, J.R., et al., *High- versus low-meat diets: effects on zinc absorption, iron status, and calcium, copper, iron, magnesium, manganese, nitrogen, phosphorus, and zinc balance in postmenopausal women.* Am J Clin Nutr, 1995. **62**(3): p. 621-32.
108. Pak, C.Y., *Pharmacotherapy of kidney stones.* Expert Opin Pharmacother, 2008. **9**(9): p. 1509-18.
109. Otsuki, M., et al., *Association of urine acidification with visceral obesity and the metabolic syndrome.* Endocr J, 2011. **58**(5): p. 363-7.
110. Hannan, M.T., et al., *Effect of dietary protein on bone loss in elderly men and women: the Framingham Osteoporosis Study.* J Bone Miner Res, 2000. **15**(12): p. 2504-12.
111. Gaffney-Stomberg, E., et al., *Increasing dietary protein requirements in elderly people for optimal muscle and bone health.* J Am Geriatr Soc, 2009. **57**(6): p. 1073-9.
112. Kerstetter, J.E., et al., *The impact of dietary protein on calcium absorption and kinetic measures of bone turnover in women.* J Clin Endocrinol Metab, 2005. **90**(1): p. 26-31.
113. Elango, R., R.O. Ball, and P.B. Pencharz, *Recent advances in determining protein and amino acid requirements in humans.* Br J Nutr, 2012. **108 Suppl 2**: p. S22-30.

114. Elango, R., et al., *Evidence that protein requirements have been significantly underestimated.* Curr Opin Clin Nutr Metab Care, 2010. **13**(1): p. 52-7.
115. Bailey, D.A., et al., *A six-year longitudinal study of the relationship of physical activity to bone mineral accrual in growing children: the university of Saskatchewan bone mineral accrual study.* J Bone Miner Res, 1999. **14**(10): p. 1672-9.
116. Gordon, C.L., et al., *The contributions of growth and puberty to peak bone mass.* Growth Dev Aging, 1991. **55**(4): p. 257-62.
117. Geinoz, G., et al., *Relationship between bone mineral density and dietary intakes in the elderly.* Osteoporos Int, 1993. **3**(5): p. 242-8.
118. Arjmandi, B.H., et al., *One year soy protein supplementation has positive effects on bone formation markers but not bone density in postmenopausal women.* Nutr J, 2005. **4**: p. 8.
119. Beasley, J.M., et al., *Is protein intake associated with bone mineral density in young women?* Am J Clin Nutr, 2010. **91**(5): p. 1311-6.
120. Darling, A.L., et al., *Dietary protein and bone health: a systematic review and meta-analysis.* Am J Clin Nutr, 2009. **90**(6): p. 1674-92.
121. Rapuri, P.B., J.C. Gallagher, and V. Haynatzka, *Protein intake: effects on bone mineral density and the rate of bone loss in elderly women.* Am J Clin Nutr, 2003. **77**(6): p. 1517-25.
122. Kerstetter, J.E., K.O. O'Brien, and K.L. Insogna, *Dietary protein, calcium metabolism, and skeletal homeostasis revisited.* Am J Clin Nutr, 2003. **78**(3 Suppl): p. 584S-592S.
123. Kerstetter, J.E., K. O'Brien, and K. Insogna, *Dietary protein and intestinal calcium absorption.* Am J Clin Nutr, 2001. **73**(5): p. 990-2.
124. Pye, K.M., et al., *A high mixed protein diet reduces body fat without altering the mechanical properties of bone in female rats.* J Nutr, 2009. **139**(11): p. 2099-105.
125. Mohan, S., et al., *Studies on regulation of insulin-like growth factor binding protein (IGFBP)-3 and IGFBP-4 production in human bone cells.* Acta Endocrinol (Copenh), 1992. **127**(6): p. 555-64.
126. Langdahl, B.L., et al., *The effects of IGF-I and IGF-II on proliferation and differentiation of human osteoblasts and interactions with growth hormone.* Eur J Clin Invest, 1998. **28**(3): p. 176-83.
127. Rubin, J., et al., *IGF-I regulates osteoprotegerin (OPG) and receptor activator of nuclear factor-kappaB ligand in vitro and OPG in vivo.* J Clin Endocrinol Metab, 2002. **87**(9): p. 4273-9.
128. Arjmandi, B.H., et al., *Soy protein has a greater effect on bone in postmenopausal women not on hormone replacement therapy, as evidenced by reducing bone resorption and urinary calcium excretion.* J Clin Endocrinol Metab, 2003. **88**(3): p. 1048-54.
129. Dawson-Hughes, B., *Interaction of dietary calcium and protein in bone health in humans.* J Nutr, 2003. **133**(3): p. 852S-854S.
130. Ceglia, L., et al., *Potassium bicarbonate attenuates the urinary nitrogen excretion that accompanies an increase in dietary protein and may promote calcium absorption.* J Clin Endocrinol Metab, 2009. **94**(2): p. 645-53.
131. Heaney, R.P., *Protein intake and bone health: the influence of belief systems on the conduct of nutritional science.* Am J Clin Nutr, 2001. **73**(1): p. 5-6.
132. Heaney, R.P., *Protein and calcium: antagonists or synergists?* Am J Clin Nutr, 2002. **75**(4): p. 609-10.

133. Calvez, J., et al., *Protein intake, calcium balance and health consequences.* Eur J Clin Nutr, 2011.
134. Heaney, R.P., *Dairy and bone health.* J Am Coll Nutr, 2009. **28 Suppl 1**: p. 82S-90S.
135. Wolfe, R.R., *The underappreciated role of muscle in health and disease.* Am J Clin Nutr, 2006. **84**(3): p. 475-82.
136. Heaney, R.P. and D.K. Layman, *Amount and type of protein influences bone health.* Am J Clin Nutr, 2008. **87**(5): p. 1567S-1570S.
137. Dawson-Hughes, B. and S.S. Harris, *Calcium intake influences the association of protein intake with rates of bone loss in elderly men and women.* Am J Clin Nutr, 2002. **75**(4): p. 773-9.
138. Conigrave, A.D., E.M. Brown, and R. Rizzoli, *Dietary protein and bone health: roles of amino acid-sensing receptors in the control of calcium metabolism and bone homeostasis.* Annu Rev Nutr, 2008. **28**: p. 131-55.
139. Cao, J.J. and F.H. Nielsen, *Acid diet (high-meat protein) effects on calcium metabolism and bone health.* Curr Opin Clin Nutr Metab Care, 2010. **13**(6): p. 698-702.
140. Kerstetter, J.E., A.M. Kenny, and K.L. Insogna, *Dietary protein and skeletal health: a review of recent human research.* Curr Opin Lipidol, 2011. **22**(1): p. 16-20.
141. Bonjour, J.P., *Protein intake and bone health.* Int J Vitam Nutr Res, 2011. **81**(2-3): p. 134-42.
142. Zou, Z.Y., et al., *Evaluation of milk basic protein supplementation on bone density and bone metabolism in Chinese young women.* Eur J Nutr, 2009. **48**(5): p. 301-6.
143. Lopez-Gonzalez, A.A., et al., *Phytate (myo-inositol hexaphosphate) and risk factors for osteoporosis.* J Med Food, 2008. **11**(4): p. 747-52.
144. Thorpe, M.P. and E.M. Evans, *Dietary protein and bone health: harmonizing conflicting theories.* Nutrition Reviews, 2011. **69**(4): p. 215-230.
145. Frassetto, L.A., et al., *Worldwide incidence of hip fracture in elderly women: relation to consumption of animal and vegetable foods.* J Gerontol A Biol Sci Med Sci, 2000. **55**(10): p. M585-92.
146. Weikert, C., et al., *The relation between dietary protein, calcium and bone health in women: results from the EPIC-Potsdam cohort.* Ann Nutr Metab, 2005. **49**(5): p. 312-8.
147. Krissansen, G.W., *Emerging health properties of whey proteins and their clinical implications.* J Am Coll Nutr, 2007. **26**(6): p. 713S-23S.
148. Tovar, A.R., et al., *A soy protein diet alters hepatic lipid metabolism gene expression and reduces serum lipids and renal fibrogenic cytokines in rats with chronic nephrotic syndrome.* J Nutr, 2002. **132**(9): p. 2562-9.
149. Mateos-Aparicio, I., et al., *Soybean, a promising health source.* Nutr Hosp, 2008. **23**(4): p. 305-12.
150. Budek, A.Z., et al., *Dietary protein intake and bone mineral content in adolescents-The Copenhagen Cohort Study.* Osteoporos Int, 2007. **18**(12): p. 1661-7.
151. Uenishi, K., et al., *Milk basic protein increases bone mineral density and improves bone metabolism in healthy young women.* Osteoporos Int, 2007. **18**(3): p. 385-90.

152. Yamamura, J., et al., *Milk basic protein (MBP) increases radial bone mineral density in healthy adult women.* Biosci Biotechnol Biochem, 2002. **66**(3): p. 702-4.
153. Xu, R., *Effect of whey protein on the proliferation and differentiation of osteoblasts.* J Dairy Sci, 2009. **92**(7): p. 3014-8.
154. Du, B., W.Z. Chai, and X.M. Lin, *[Effect of the whey basic protein on bone mineral density]*. Zhonghua Yu Fang Yi Xue Za Zhi, 2007. **41**(2): p. 96-100.
155. Alekel, D.L., et al., *Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women.* Am J Clin Nutr, 2000. **72**(3): p. 844-52.
156. Kreijkamp-Kaspers, S., et al., *Effect of soy protein containing isoflavones on cognitive function, bone mineral density, and plasma lipids in postmenopausal women: a randomized controlled trial.* JAMA, 2004. **292**(1): p. 65-74.
157. Gallagher, J.C., et al., *The effect of soy protein isolate on bone metabolism.* Menopause, 2004. **11**(3): p. 290-8.
158. Reinwald, S. and C.M. Weaver, *Soy isoflavones and bone health: a double-edged sword?* J Nat Prod, 2006. **69**(3): p. 450-9.
159. Zhang, X., et al., *Prospective cohort study of soy food consumption and risk of bone fracture among postmenopausal women.* Arch Intern Med, 2005. **165**(16): p. 1890-5.
160. Zhou, Y., et al., *The effect of soy food intake on mineral status in premenopausal women.* J Womens Health (Larchmt), 2011. **20**(5): p. 771-80.
161. Hsu, C.S., et al., *Soy isoflavone supplementation in postmenopausal women. Effects on plasma lipids, antioxidant enzyme activities and bone density.* J Reprod Med, 2001. **46**(3): p. 221-6.
162. Zhang, J., et al., *Differential effects of short term feeding of a soy protein isolate diet and estrogen treatment on bone in the pre-pubertal rat.* PLoS One, 2012. **7**(4): p. e35736.
163. Ho, S.C., et al., *Soy protein consumption and bone mass in early postmenopausal Chinese women.* Osteoporos Int, 2003. **14**(10): p. 835-42.
164. Horiuchi, T., et al., *Effect of soy protein on bone metabolism in postmenopausal Japanese women.* Osteoporos Int, 2000. **11**(8): p. 721-4.
165. Setchell, K.D., et al., *S-equol, a potent ligand for estrogen receptor beta, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora.* Am J Clin Nutr, 2005. **81**(5): p. 1072-9.
166. Roughead, Z.K., et al., *Controlled substitution of soy protein for meat protein: effects on calcium retention, bone, and cardiovascular health indices in postmenopausal women.* J Clin Endocrinol Metab, 2005. **90**(1): p. 181-9.
167. Potter, S.M., et al., *Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women.* Am J Clin Nutr, 1998. **68**(6 Suppl): p. 1375S-1379S.
168. Spence, L.A., et al., *The effect of soy protein and soy isoflavones on calcium metabolism in postmenopausal women: a randomized crossover study.* Am J Clin Nutr, 2005. **81**(4): p. 916-22.
169. Rowland, I.R., et al., *Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora.* Nutr Cancer, 2000. **36**(1): p. 27-32.
170. Chen, J.R., et al., *Short term effects on bone quality associated with consumption of soy protein isolate and other dietary protein sources in rapidly growing female rats.* Exp Biol Med (Maywood), 2008. **233**(11): p. 1348-58.

171. Newton, K.M., et al., *Soy protein and bone mineral density in older men and women: a randomized trial.* Maturitas, 2006. **55**(3): p. 270-7.
172. Borer, K.T., *Physical activity in the prevention and amelioration of osteoporosis in women : interaction of mechanical, hormonal and dietary factors.* Sports Med, 2005. **35**(9): p. 779-830.
173. Janz, K.F., et al., *Physical activity and femoral neck bone strength during childhood: the Iowa Bone Development Study.* Bone, 2007. **41**(2): p. 216-22.
174. Sone, T., et al., *Side-to-side differences in cortical bone mineral density of tibiae in young male athletes.* Bone, 2006. **38**(5): p. 708-13.
175. Falk, B., et al., *A cumulative effect of physical training on bone strength in males.* Int J Sports Med, 2007. **28**(6): p. 449-55.
176. Daly, R.M. and S.L. Bass, *Lifetime sport and leisure activity participation is associated with greater bone size, quality and strength in older men.* Osteoporos Int, 2006. **17**(8): p. 1258-67.
177. Nurzenski, M.K., et al., *Geometric indices of bone strength are associated with physical activity and dietary calcium intake in healthy older women.* J Bone Miner Res, 2007. **22**(3): p. 416-24.
178. Umemura, Y., et al., *A time course of bone response to jump exercise in C57BL/6J mice.* J Bone Miner Metab, 2002. **20**(4): p. 209-15.
179. Hamrick, M.W., et al., *Increased osteogenic response to exercise in metaphyseal versus diaphyseal cortical bone.* J Musculoskelet Neuronal Interact, 2006. **6**(3): p. 258-63.
180. Ma, H., et al., *Effects of diet-induced obesity and voluntary wheel running on bone properties in young male C57BL/6J mice.* Calcif Tissue Int, 2010. **86**(5): p. 411-9.
181. Aparicio, V.A., et al., *Effects of high-whey-protein intake and resistance training on renal, bone and metabolic parameters in rats.* Br J Nutr, 2011. **105**(6): p. 836-45.
182. Bennell, K., et al., *Effects of resistance training on bone parameters in young and mature rats.* Clin Exp Pharmacol Physiol, 2000. **27**(1-2): p. 88-94.
183. Burr, D.B., A.G. Robling, and C.H. Turner, *Effects of biomechanical stress on bones in animals.* Bone, 2002. **30**(5): p. 781-6.
184. Gustavsson, A., K. Thorsen, and P. Nordstrom, *A 3-year longitudinal study of the effect of physical activity on the accrual of bone mineral density in healthy adolescent males.* Calcif Tissue Int, 2003. **73**(2): p. 108-14.
185. Bailey, D.A., et al., *Calcium accretion in girls and boys during puberty: a longitudinal analysis.* J Bone Miner Res, 2000. **15**(11): p. 2245-50.
186. Karlsson, M.K., et al., *The duration of exercise as a regulator of bone mass.* Bone, 2001. **28**(1): p. 128-32.
187. Gomez-Ambrosi, J., et al., *The bone-adipose axis in obesity and weight loss.* Obes Surg, 2008. **18**(9): p. 1134-43.
188. Pollock, N.K., et al., *Is adiposity advantageous for bone strength? A peripheral quantitative computed tomography study in late adolescent females.* Am J Clin Nutr, 2007. **86**(5): p. 1530-8.
189. Reid, I.R., *Relationships between fat and bone.* Osteoporos Int, 2008. **19**(5): p. 595-606.
190. Judex S, R.J., Rubin CT., *Mechanism of exercise effects on bone quantity and quality*, in *Principles of Bone Biology*, J.P. Bilezikian, Raisz, L. G., Martin, T. J., Editor. 2008. p. 1819-1837.

191. Li, Y.J., et al., *Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation.* J Orthop Res, 2004. **22**(6): p. 1283-9.
192. Rubin, J., et al., *Activation of extracellular signal-regulated kinase is involved in mechanical strain inhibition of RANKL expression in bone stromal cells.* J Bone Miner Res, 2002. **17**(8): p. 1452-60.
193. Wiltink, A., et al., *Cell membrane stretch in osteoclasts triggers a self-reinforcing Ca2+ entry pathway.* Pflugers Arch, 1995. **429**(5): p. 663-71.
194. Boo, Y.C. and H. Jo, *Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases.* Am J Physiol Cell Physiol, 2003. **285**(3): p. C499-508.
195. Rubin, J., et al., *Mechanical strain differentially regulates endothelial nitric-oxide synthase and receptor activator of nuclear kappa B ligand expression via ERK1/2 MAPK.* J Biol Chem, 2003. **278**(36): p. 34018-25.
196. Skerry, T.M., et al., *Early strain-related changes in enzyme activity in osteocytes following bone loading in vivo.* J Bone Miner Res, 1989. **4**(5): p. 783-8.
197. Inaoka, T., et al., *Sequential analysis of gene expression after an osteogenic stimulus: c-fos expression is induced in osteocytes.* Biochem Biophys Res Commun, 1995. **217**(1): p. 264-70.
198. Ehrlich, P.J. and L.E. Lanyon, *Mechanical strain and bone cell function: a review.* Osteoporos Int, 2002. **13**(9): p. 688-700.
199. Tipton, K.D., *Efficacy and consequences of very-high-protein diets for athletes and exercisers.* Proc Nutr Soc, 2011. **70**(2): p. 205-14.
200. Babraj, J.A., et al., *Human bone collagen synthesis is a rapid, nutritionally modulated process.* J Bone Miner Res, 2005. **20**(6): p. 930-7.
201. Wu, J., et al., *Combined intervention of soy isoflavone and moderate exercise prevents body fat elevation and bone loss in ovariectomized mice.* Metabolism, 2004. **53**(7): p. 942-8.
202. Oh, H.Y., et al., *A combination of soy isoflavone supplementation and exercise improves lipid profiles and protects antioxidant defense-systems against exercise-induced oxidative stress in ovariectomized rats.* Biofactors, 2007. **29**(4): p. 175-85.
203. Tomayko, E.J., H.R. Chung, and K.R. Wilund, *Soy protein diet and exercise training increase relative bone volume and enhance bone microarchitecture in a mouse model of uremia.* J Bone Miner Metab, 2011. **29**(6): p. 682-90.
204. Chilibeck, P.D. and S.M. Cornish, *Effect of estrogenic compounds (estrogen or phytoestrogens) combined with exercise on bone and muscle mass in older individuals.* Appl Physiol Nutr Metab, 2008. **33**(1): p. 200-12.
205. Welch, J.M. and C.M. Weaver, *Calcium and exercise affect the growing skeleton.* Nutr Rev, 2005. **63**(11): p. 361-73.
206. Specker, B.L., *Evidence for an interaction between calcium intake and physical activity on changes in bone mineral density.* J Bone Miner Res, 1996. **11**(10): p. 1539-44.
207. Gunnes, M. and E.H. Lehmann, *Physical activity and dietary constituents as predictors of forearm cortical and trabecular bone gain in healthy children and adolescents: a prospective study.* Acta Paediatr, 1996. **85**(1): p. 19-25.
208. Specker, B. and T. Binkley, *Randomized trial of physical activity and calcium supplementation on bone mineral content in 3- to 5-year-old children.* J Bone Miner Res, 2003. **18**(5): p. 885-92.

209. Specker, B., T. Binkley, and N. Fahrenwald, *Increased periosteal circumference remains present 12 months after an exercise intervention in preschool children.* Bone, 2004. **35**(6): p. 1383-8.
210. McDonald, R., J. Hegenauer, and P. Saltman, *Age-related differences in the bone mineralization pattern of rats following exercise.* J Gerontol, 1986. **41**(4): p. 445-52.
211. Karcioglu Z.A. and S. R.M., *Zinc and Copper in Medicine.*, ed. C.C.T. Publ. 1980, Springfield.
212. Vicente-Rodriguez, G., et al., *Independent and combined effect of nutrition and exercise on bone mass development.* J Bone Miner Metab, 2008. **26**(5): p. 416-24.
213. Melchert, R.B. and A.A. Welder, *Cardiovascular effects of androgenic-anabolic steroids.* Med Sci Sports Exerc, 1995. **27**(9): p. 1252-62.
214. Casner, S.W., Jr., R.G. Early, and B.R. Carlson, *Anabolic steroid effects on body composition in normal young men.* J Sports Med Phys Fitness, 1971. **11**(2): p. 98-103.
215. Friedl, K.E., et al., *Comparison of the effects of high dose testosterone and 19-nortestosterone to a replacement dose of testosterone on strength and body composition in normal men.* J Steroid Biochem Mol Biol, 1991. **40**(4-6): p. 607-12.
216. Hartgens, F., et al., *Androgenic-anabolic steroid-induced body changes in strength athletes.* Phys Sportsmed, 2001. **29**(1): p. 49-65.
217. Kuipers, H., et al., *Influence of anabolic steroids on body composition, blood pressure, lipid profile and liver functions in body builders.* Int J Sports Med, 1991. **12**(4): p. 413-8.
218. Yeater, R., et al., *Resistance trained athletes using or not using anabolic steroids compared to runners: effects on cardiorespiratory variables, body composition, and plasma lipids.* Br J Sports Med, 1996. **30**(1): p. 11-4.
219. Nordstrom, A., et al., *Higher muscle mass but lower gynoid fat mass in athletes using anabolic androgenic steroids.* J Strength Cond Res, 2012. **26**(1): p. 246-50.
220. Wiren, K.M., *Androgens Receptor Expression and Steroid Action in Bone*, in *Principles of Bone Biology*, J.P. Bilezikian, Lawrence G. Raisz, T. John Martin, Editor. 2008. p. 1001-1018.
221. Kicman, A.T., *Pharmacology of anabolic steroids.* Br J Pharmacol, 2008. **154**(3): p. 502-21.
222. Nawata, H., et al., *Aromatase in bone cell: association with osteoporosis in postmenopausal women.* J Steroid Biochem Mol Biol, 1995. **53**(1-6): p. 165-74.
223. Sasano, H., et al., *Aromatase in human bone tissue.* J Bone Miner Res, 1997. **12**(9): p. 1416-23.
224. Schweikert, H.U., L. Wolf, and G. Romalo, *Oestrogen formation from androstenedione in human bone.* Clin Endocrinol (Oxf), 1995. **43**(1): p. 37-42.
225. Nakano, Y., et al., *The receptor, metabolism and effects of androgen in osteoblastic MC3T3-E1 cells.* Bone Miner, 1994. **26**(3): p. 245-59.
226. Jones, M.E., et al., *Recognizing rare disorders: aromatase deficiency.* Nat Clin Pract Endocrinol Metab, 2007. **3**(5): p. 414-21.
227. Hartgens, F. and H. Kuipers, *Effects of androgenic-anabolic steroids in athletes.* Sports Med, 2004. **34**(8): p. 513-54.
228. Wyllie, A.H., J.F. Kerr, and A.R. Currie, *Cell death: the significance of apoptosis.* Int Rev Cytol, 1980. **68**: p. 251-306.

229. Wiren, K.M., et al., *Osteoblast and osteocyte apoptosis associated with androgen action in bone: requirement of increased Bax/Bcl-2 ratio.* Bone, 2006. **38**(5): p. 637-51.
230. Gruber, R., et al., *Expression of the vitamin D receptor, of estrogen and thyroid hormone receptor alpha- and beta-isoforms, and of the androgen receptor in cultures of native mouse bone marrow and of stromal/osteoblastic cells.* Bone, 1999. **24**(5): p. 465-73.
231. Sinha-Hikim, I., et al., *Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment.* J Clin Endocrinol Metab, 2004. **89**(10): p. 5245-55.
232. Herbst, K.L. and S. Bhasin, *Testosterone action on skeletal muscle.* Curr Opin Clin Nutr Metab Care, 2004. **7**(3): p. 271-7.
233. Michael, H., et al., *Estrogen and testosterone use different cellular pathways to inhibit osteoclastogenesis and bone resorption.* J Bone Miner Res, 2005. **20**(12): p. 2224-32.
234. Francis, R.M., *The effects of testosterone on osteoporosis in men.* Clin Endocrinol (Oxf), 1999. **50**(4): p. 411-4.
235. Wimalawansa, S.M., et al., *Reversal of weightlessness-induced musculoskeletal losses with androgens: quantification by MRI.* J Appl Physiol, 1999. **86**(6): p. 1841-6.
236. Schot, L.P., A.H. Schuurs, and H.J. Kloosterboer, *The action of anabolic steroids on bone in experimental animals.* Wien Med Wochenschr, 1993. **143**(14-15): p. 385-7.
237. Nuti, R., et al., *[Effects of nandrolone decanoate on bone mineral content and intestinal absorption of calcium]*. Minerva Med, 1984. **75**(3-4): p. 109-13.
238. Li, X., et al., *The effects of nandrolone decanoate on bone mass and metabolism in ovariectomized rats with osteopenia.* J Bone Miner Metab, 2000. **18**(5): p. 258-63.
239. Aithal, H.P., et al., *Effects of Nandrolone and TGF-beta1 in growing rabbits with osteopenia induced by over-supplementation of calcium and vitamin D3.* Vet Res Commun, 2009. **33**(4): p. 331-43.
240. Geusens, P. and J. Dequeker, *Long-term effect of nandrolone decanoate, 1 alpha-hydroxyvitamin D3 or intermittent calcium infusion therapy on bone mineral content, bone remodeling and fracture rate in symptomatic osteoporosis: a double-blind controlled study.* Bone Miner, 1986. **1**(4): p. 347-57.
241. Hassager, C., et al., *Nandrolone decanoate treatment of post-menopausal osteoporosis for 2 years and effects of withdrawal.* Maturitas, 1989. **11**(4): p. 305-17.
242. Adami, S., et al., *The prevention of corticosteroid-induced osteoporosis with nandrolone decanoate.* Bone Miner, 1991. **15**(1): p. 73-81.
243. Johansen, J.S., et al., *Treatment of postmenopausal osteoporosis: is the anabolic steroid nandrolone decanoate a candidate?* Bone Miner, 1989. **6**(1): p. 77-86.
244. Need, A.G., et al., *Effects of nandrolone decanoate and antiresorptive therapy on vertebral density in osteoporotic postmenopausal women.* Arch Intern Med, 1989. **149**(1): p. 57-60.
245. Geusens, P., et al., *Calcium-deficient diet in ovariectomized dogs limits the effects of 17 beta-estradiol and nandrolone decanoate on bone.* J Bone Miner Res, 1991. **6**(8): p. 791-7.
246. Geusens, P., et al., *Heterogeneity of growth of bone in children at the spine, radius and total skeleton.* Growth Dev Aging, 1991. **55**(4): p. 249-56.

247. Aerssens, J., et al., *Mechanical properties, bone mineral content, and bone composition (collagen, osteocalcin, IGF-I) of the rat femur: influence of ovariectomy and nandrolone decanoate (anabolic steroid) treatment.* Calcif Tissue Int, 1993. **53**(4): p. 269-77.
248. Estoppey-Stojanovski, L., *[Position of the Council of Europe on the protection of animals]*. Dev Biol Stand, 1986. **64**: p. 3-5.
249. Reeves, P.G., F.H. Nielsen, and G.C. Fahey, Jr., *AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet.* J Nutr, 1993. **123**(11): p. 1939-51.
250. de Salles, B.F., et al., *Rest interval between sets in strength training.* Sports Med, 2009. **39**(9): p. 765-77.
251. Schneider, M.R., et al., *High cortical bone mass phenotype in betacellulin transgenic mice is EGFR dependent.* J Bone Miner Res, 2009. **24**(3): p. 455-67.
252. Pichon, L., et al., *High-protein diets containing different milk protein fractions differently influence energy intake and adiposity in the rat.* Br J Nutr, 2008. **99**(4): p. 739-48.
253. Belobrajdic, D.P., G.H. McIntosh, and J.A. Owens, *A high-whey-protein diet reduces body weight gain and alters insulin sensitivity relative to red meat in wistar rats.* J Nutr, 2004. **134**(6): p. 1454-8.
254. Bouthegourd, J.C., et al., *A preexercise alpha-lactalbumin-enriched whey protein meal preserves lipid oxidation and decreases adiposity in rats.* Am J Physiol Endocrinol Metab, 2002. **283**(3): p. E565-72.
255. Johnson, D.J. and G.H. Anderson, *Prediction of plasma amino acid concentration from diet amino acid content.* Am J Physiol, 1982. **243**(1): p. R99-103.
256. Anderson GH, G.N., Li E.T.S., *Amino acids and the regulation of quantitative and qualitative aspects of food intake.* Amino Acids: Metabolism and Medical Applications, 1983: p. 225-238.
257. Tews, J.K., S.S. Good, and A.E. Harper, *Transport of threonine and tryptophan by rat brain slices: relation to other amino acids at concentrations found in plasma.* J Neurochem, 1978. **31**(3): p. 581-9.
258. Aparicio, V.A., et al., *[Resistance training reduces the metabolic acidosis and hepatic and renal hypertrophy caused by the consumption of a high protein diet in rats]*. Nutr Hosp, 2011. **26**(6): p. 1478-86.
259. V. A. Aparicio, E.N., R. García-del Moral, M. Machado-Vílchez, J. M. Porres, C. Sánchez and P. Aranda, *High-protein diets and renal status in rats.* Nutrición Hospitalaria, 2013. **28**(1): p. 232-237.
260. Kaji M, N.Y., *Growth and Minerals: Zinc.* Growth, Genetics & Hormones, 2006. **22**(1): p. 1-7.
261. Zhou, J., et al., *Dietary whey protein decreases food intake and body fat in rats.* Obesity (Silver Spring), 2011. **19**(8): p. 1568-73.
262. Yu, Y., T. South, and X.F. Huang, *Inter-meal interval is increased in mice fed a high whey, as opposed to soy and gluten, protein diets.* Appetite, 2009. **52**(2): p. 372-9.
263. Eller, L.K. and R.A. Reimer, *Dairy protein attenuates weight gain in obese rats better than whey or casein alone.* Obesity (Silver Spring), 2010. **18**(4): p. 704-11.

264. Walzem, R.L., C.J. Dillard, and J.B. German, *Whey components: millennia of evolution create functionalities for mammalian nutrition: what we know and what we may be overlooking.* Crit Rev Food Sci Nutr, 2002. **42**(4): p. 353-75.
265. Yudkoff, M., et al., *Brain amino acid requirements and toxicity: the example of leucine.* J Nutr, 2005. **135**(6 Suppl): p. 1531S-8S.
266. Morrison, C.D., et al., *Amino acids inhibit Agrp gene expression via an mTOR-dependent mechanism.* Am J Physiol Endocrinol Metab, 2007. **293**(1): p. E165-71.
267. Aparicio, V.N., E.; Heredia, JM.; Aranda, P., *Efectos metabólicos, renales y óseos de las dietas hiperproteicas. Papel regulador del ejercicio.* Revista Andaluza de Medicina del Deporte, 2010. **3**(4): p. 153-158.
268. Kenny, A.M., et al., *Soy proteins and isoflavones affect bone mineral density in older women: a randomized controlled trial123.* Am J Clin Nutr, 2009. **90**(1): p. 234-42.
269. Horiuchi, T., *[Soy protein intake and bone mineral density]*. Clin Calcium, 2005. **15**(9): p. 1507-13.
270. Houston, M.C., et al., *Nonpharmacologic treatment of dyslipidemia.* Prog Cardiovasc Dis, 2009. **52**(2): p. 61-94.
271. Williams, M.A., et al., *Resistance exercise in individuals with and without cardiovascular disease: 2007 update: a scientific statement from the American Heart Association Council on Clinical Cardiology and Council on Nutrition, Physical Activity, and Metabolism.* Circulation, 2007. **116**(5): p. 572-84.
272. Tremblay, A., J.P. Despres, and C. Bouchard, *The effects of exercise-training on energy balance and adipose tissue morphology and metabolism.* Sports Med, 1985. **2**(3): p. 223-33.
273. Morimoto, Y., K. Arisue, and Y. Yamamura, *Relationship between circadian rhythm of food intake and that of plasma corticosterone and effect of food restriction on circadian adrenocortical rhythm in the rat.* Neuroendocrinology, 1977. **23**(4): p. 212-22.
274. Stump, C.S., *Physical Activity in the Prevention of Chronic Kidney Disease.* Cardiorenal Med, 2011. **1**(3): p. 164-173.
275. Moinuddin, I. and D.J. Leehey, *A comparison of aerobic exercise and resistance training in patients with and without chronic kidney disease.* Adv Chronic Kidney Dis, 2008. **15**(1): p. 83-96.
276. Poortmans, J.R. and M. Ouchinsky, *Glomerular filtration rate and albumin excretion after maximal exercise in aging sedentary and active men.* J Gerontol A Biol Sci Med Sci, 2006. **61**(11): p. 1181-5.
277. Aparicio, V.A., *Effects of the amount and source of protein, resistance training and steroids on metabolic, hepatic, renal and bone markers in rats*, in *Physiology*. 2012, Granada: Granada. p. 188.
278. Usui, Y., et al., *Urinary citrate in kidney stone disease.* Tokai J Exp Clin Med, 2003. **28**(2): p. 65-70.
279. Cossio-Bolanos MA, G.-C.R., Pilco-Quesada S, Lancho-Alonso JL, de Arruda M, *Propuesta de una ecuación lineal para valorar la velocidad de crecimiento somático a partir de la masa corporal de ratas machos Wistar.* Anales de la Facultad de Medicina, 2012. **73**(2): p. 93-100.
280. Hughes, P.C. and J.M. Tanner, *The assessment of skeletal maturity in the growing rat.* J Anat, 1970. **106**(Pt 2): p. 371-402.

281. Bailey, D.A., Malina, R.M., Rasmussen, R.M., *The influence of exercise, physical activity and athletic performance on the dynamics effects of human growth*, in *Human growth*, F.a. Tanner, Editor. 1978. p. 475-505.
282. Gutierrez, A., *Modificaciones fisiológicas inducidas por el ejercicio en adolescentes y su repercusión sobre la talla definitiva*, in *Archivos de Medicina del Deporte*. 1989. p. 265-268.
283. Pagani, F., C.M. Francucci, and L. Moro, *Markers of bone turnover: biochemical and clinical perspectives.* J Endocrinol Invest, 2005. **28**(10 Suppl): p. 8-13.
284. Gade, T.P., et al., *Imaging of alkaline phosphatase activity in bone tissue.* PLoS One, 2011. **6**(7): p. e22608.
285. Smith, S.M., et al., *Benefits for bone from resistance exercise and nutrition in long-duration spaceflight: Evidence from biochemistry and densitometry.* J Bone Miner Res, 2012. **27**(9): p. 1896-906.
286. Alsio, J., et al., *Impact of nandrolone decanoate on gene expression in endocrine systems related to the adverse effects of anabolic androgenic steroids.* Basic Clin Pharmacol Toxicol, 2009. **105**(5): p. 307-14.
287. Spitz, I.M., et al., *Effect of non aromatizable androgens on LHRH and TRH responses in primary testicular failure.* Horm Metab Res, 1984. **16**(9): p. 492-7.
288. Schlussman, S.D., et al., *Effects of the androgenic anabolic steroid, nandrolone decanoate, on adrenocorticotropin hormone, corticosterone and proopiomelanocortin, corticotropin releasing factor (CRF) and CRF receptor1 mRNA levels in the hypothalamus, pituitary and amygdala of the rat.* Neurosci Lett, 2000. **284**(3): p. 190-4.

VIII. TABLES AND FIGURES

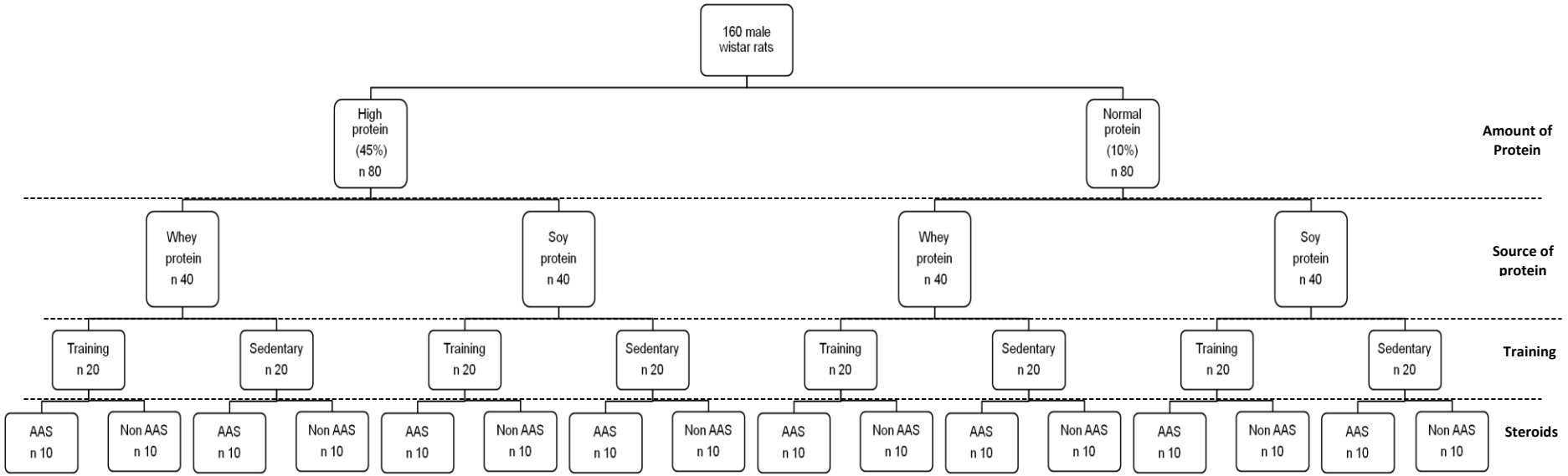

Figure 1. Study design showing the four different interventions: dietary protein amount (high-protein vs. normal-protein), protein source (whey vs. soy), training (resistance training vs. sedentary) and anabolic-androgenic steroids (AAS) (with AAS-administration vs. without AAS-administration).

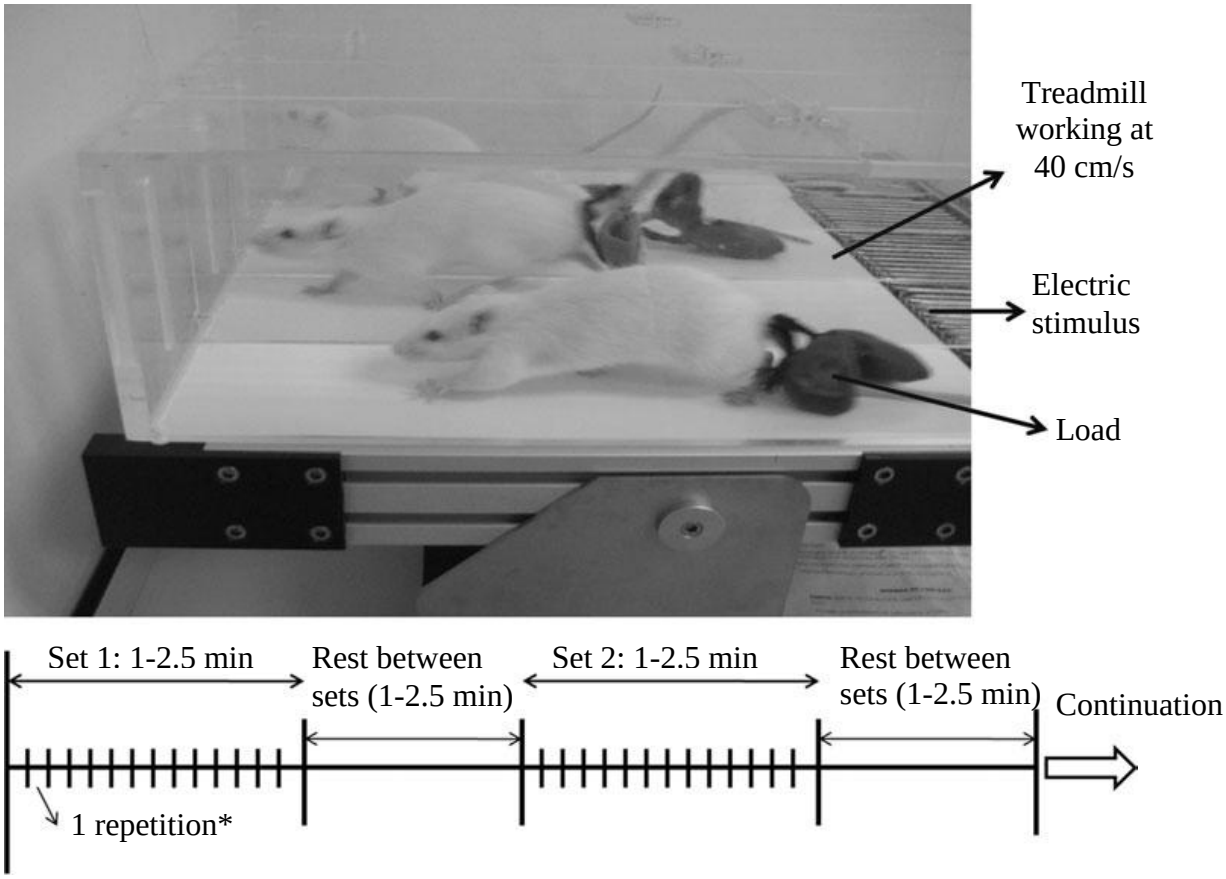

Figure 2. Rats training on the treadmill and details of the resistance training protocol. Number of sets per training session: 10 – 12. Number of repetitions per set: 8 – 14.
*A repetition is done when the resistance is transported along 20 – 40cm or 2 – 4s pulling the resistance although no distance is achieved.

Table 3. Mineral composition of the experimental diets

Mineral Composition	**Whey protein diet**		**Soy protein diet**	
	Normal-protein	***High-protein***	***Normal-protein***	***High-protein***
Diet dry matter content (g)	0.62 (0.07)	0.57 (0.03)	0.59 (0.04)	0.55 (0.06)
Diet ash content (g)	0.016 (0.002)	0.025 (0.001)	0.021 (0.001)	0.028 (0.003)
Diet Ca content (mg/g DM)	5.39 (0.23)	5.48 (0.24)	6.08 (0.76)	7.52 (0.08)
Diet P content (mg/g DM)	2.50 (0.10)	3.18 (0.15)	3.63 (0.13)	6.25 (0.13)
Diet Mg content (mg/g DM)	0.52 (0.02)	0.56 (0.01)	0.49 (0.02)	0.83 (0.01)
Diet Zn content (µg/g DM)	29.33 (1.27)	28.07 (0.70)	23.0 (0.15)	41.66 (1.02)

DM, dry matter. Values expressed as mean (standard error of the mean).

Table 4. Effects of the protein diet concentration, source of protein, resistance training and anabolic-androgenic steroids (AAS) on diet intake, protein intake, weight increase, protein efficiency ratio and food transformation ratio *.

	Protein diet concentration			Source of protein			Exercise			AAS		
	Normal protein (10%)	High protein (45%)	P	Animal (whey)	Vegetal (soy)	P	Sedentary	Resistance training	P	No AAS	AAS	P
Diet intake DM (g/rat/day)	15.58(0.25)	13.73(0.21)	0.000	13.92(0.28)	15.00(0.22)	0.003	15.45(0.24)	13.62(0.21)	0.000	15.44(0.25)	13.95(0.23)	0.000
Protein intake (g/rat/day)	1.64(0.02)	6.44(0.13)	0.000	4.36(0.31)	4.21(0.30)	0.740	4.50(0.32)	4.02(0.29)	0.267	4.95(0.34)	3.79(0.27)	0.009
Δ rat weight (g/rat/day)	1.94(0.06)	1.74(0.05)	0.016	1.80(0.06)	1.85(0.05)	0.242	2.03(0.05)	1.62(0.06)	0.000	2.04(0.06)	1.68(0.05)	0.000
Protein Efficiency Ratio (PER)	1.24(0.03)	0.28(0.01)	0.000	0.62(0.06)	0.78(0.06)	0.063	0.76(0.06)	0.67(0.06)	0.001	0.68(0.07)	0.74(0.06)	0.023
Food Transformation Ratio (DM)	8.31(0.18)	8.29(0.22)	0.470	8.04(0.22)	8.47(0.19)	0.154	7.83(0.17)	8.80(0.22)	0.001	7.80(0.19)	8.65(0.20)	0.004

DM, dry matter. Δ, increase. Values expressed as mean (standard error of the mean).

* Results for 12 weeks of the experiment.

Table 5. Effects of the protein diet concentration, source of protein, resistance training and anabolic-androgenic steroids (AAS) on digestive utilization of calcium.

	Protein diet concentration			Source of protein			Exercise			AAS		
	Normal protein (10%)	High protein (45%)	P	Animal (whey)	Vegetal (soy)	P	Sedentary	Resistance training	P	No AAS	AAS	P
Diet intake DM (g/rat/day)*	16.43(0.25)	14.58(0.30)	0.000	14.25(0.35)	16.20(0.23)	0.000	16.18(0.24)	14.60(0.33)	0.000	16.33(0.23)	14.77(0.31)	0.000
Calcium intake (mg/d)	96.38(1.80)	97.15(3.09)	0.830	77.40(1.90)	109.50(1.71)	0.000	102.01(2.58)	91.27(2.53)	0.004	105.03(1.98)	91.07(2.67)	0.000
Calcium faecal (mg/d)	52.78(3.19)	65.53(2.47)	0.002	48.23(4.10)	64.36(2.18)	0.001	58.05(3.19)	59.95(2.70)	0.649	63.54(3.16)	56.60(2.73)	0.122
Absorbed calcium (mg/d)	43.33(2.76)	33.68(4.67)	0.078	25.87(4.92)	45.15(2.96)	0.001	45.37(3.93)	30.98(3.37)	0.007	47.14(2.70)	34.38(3.74)	0.007
Calcium CDA (%)	45.81(3.04)	27.81(3.64)	0.000	31.55(5.87)	39.94(2.27)	0.188	42.09(3.72)	31.41(3.09)	0.029	42.99(2.55)	34.15(3.49)	0.043

Values expressed as mean (standard error of the mean).
* Results of the last week of experience.

Table 6. Effects of the protein diet concentration, source of protein, resistance training and anabolic-androgenic steroids (AAS) on digestive utilization of magnesium.

	Protein diet concentration			Source of protein			Exercise			AAS		
	Normal protein (10%)	High protein (45%)	P	Animal (whey)	Vegetal (soy)	P	Sedentary	Resistance training	P	No AAS	AAS	P
Diet intake DM (g/rat/day)*	16.43(0.25)	14.58(0.30)	0.000	14.25(0.35)	16.20(0.23)	0.000	16.18(0.24)	14.60(0.33)	0.000	16.33(0.23)	14.77(0.31)	0.000
Magnesium intake (mg/d)	8.20(0.12)	10.45(0.37)	0.000	7.73(0.18)	10.54(0.30)	0.000	9.96(0.33)	8.87(0.30)	0.017	10.20(0.29)	8.89(0.32)	0.005
Magnesium faecal (mg/d)	3.27(0.23)	4.11(0.25)	0.014	2.01(0.20)	4.49(0.17)	0.000	3.60(0.22)	3.78(0.27)	0.608	4.38(0.23)	3.32(0.22)	0.003
Absorbed magnesium (mg/d)	4.93(0.22)	6.72(0.40)	0.000	5.31(0.26)	6.05(0.33)	0.084	6.47(0.38)	5.08(0.24)	0.003	6.27(0.37)	5.57(0.30)	0.164
Magnesium CDA (%)	60.48(2.59)	61.42(2.09)	0.778	72.22(2.54)	55.52(1.86)	0.000	62.87(2.57)	58.82(2.03)	0.219	57.96(2.55)	62.50(2.14)	0.197

Values expressed as mean (standard error of the mean).
* Results of the last week of experience

Table 7. Effects of the protein diet concentration, source of protein, resistance training and anabolic-androgenic steroids (AAS) on digestive utilization of phosphorus.

	Protein diet concentration			Source of protein			Exercise			AAS		
	Normal protein (10%)	High protein (45%)	P	Animal (whey)	Vegetal (soy)	P	Sedentary	Resistance training	P	No AAS	AAS	P
Diet intake DM (g/rat/day)*	16.43(0.25)	14.58(0.30)	0.000	14.25(0.35)	16.20(0.23)	0.000	16.18(0.24)	14.60(0.33)	0.000	16.33(0.23)	14.77(0.31)	0.000
Phosphorus intake (mg/d)	53.88(1.56)	72.10(3.41)	0.000	41.39(1.01)	78.86(2.30)	0.000	67.48(3.18)	59.99(2.78)	0.081	71.81(2.70)	58.24(2.97)	0.002
Phosphorus faecal (mg/d)	21.70(1.37)	32.95(1.76)	0.000	14.70(1.26)	33.26(1.23)	0.000	26.57(1.49)	27.96(1.98)	0.575	32.93(1.90)	24.27(1.47)	0.001
Absorbed phosphorus (mg/d)	32.19(1.21)	44.72(3.06)	0.000	23.30(1.28)	45.59(2.03)	0.000	42.87(2.68)	33.35(1.88)	0.004	46.78(1.80)	33.97(2.29)	0.000
Phosphorus CDA (%)	60.88(2.14)	55.83(1.65)	0.065	61.42(3.01)	56.94(1.41)	0.184	61.43(2.07)	55.05(1.67)	0.018	59.32(1.71)	57.92(1.89)	0.583

Values expressed as mean (standard error of the mean).
* Results of the last week of experience

Table 8. Effects of the protein diet concentration, source of protein, resistance training and anabolic-androgenic steroids (AAS) on digestive utilization of zinc.

	Protein diet concentration			Source of protein			Exercise			AAS		
	Normal protein (10%)	High protein (45%)	P	Animal (whey)	Vegetal (soy)	P	Sedentary	Resistance training	P	No AAS	AAS	P
Diet intake DM (g/rat/day)*	16.43(0.25)	14.58(0.30)	0.000	14.25(0.35)	16.20(0.23)	0.000	16.18(0.24)	14.60(0.33)	0.000	16.33(0.23)	14.77 (0.31)	0.000
Zinc intake (µg/d)	410.17(7.29)	523.21(18.24)	0.000	407.17(10.45)	515.44(16.32)	0.000	498.22(16.60)	444.03(15.42)	0.019	499.86(15.50)	452.47 (16.14)	0.043
Zinc faecal (µg/d)	229.25(13.49)	260.60(9.93)	0.064	223.35(19.29)	254.78(8.45)	0.142	262.85(13.87)	225.20(8.90)	0.024	251.89(9.61)	240.95 (11.91)	0.476
Absorbed zinc (µg/d)	180.37(14.19)	290.15(20.51)	0.000	173.95(23.96)	261.55(15.15)	0.002	248.48(19.42)	218.45(18.10)	0.261	270.60(25.84)	214.76 (14.82)	0.065
Zinc CDA (%)	43.59(3.03)	48.61(2.63)	0.215	41.39(4.92)	48.19(1.86)	0.202	46.26(3.21)	45.81(2.43)	0.910	47.57(3.34)	45.24 (2.54)	0.586

Values expressed as mean (standard error of the mean)
* Results of the last week of experience

Table 9. Effects of the protein diet concentration, source of protein, resistance training and anabolic-androgenic steroids (AAS) on final body weight, dry femur weight, femur ash and bone mineral content.

	Protein diet concentration			Source of protein			Exercise			AAS		
	Normal protein (10%)	High protein (45%)	P	Animal (whey)	Vegetal (soy)	P	Sedentary	Resistance training	P	No AAS	AAS	P
Final body weight (g)	327.33 (3.90)	324.80 (4.78)	0.749	327.8 (4.97)	324.64 (3.79)	0.362	340.63 (3.82)	310.56 (4.16)	0.000	336.10 (4.39)	316.96 (4.04)	0.002
Femur dry weight (g)	0.56 (0.006)	0.58 (0.007)	0.069	0.57 (0.007)	0.57 (0.006)	0.956	0.58 (0.006)	0.56 (0.007)	0.037	0.57 (0.007)	0.56 (0.005)	0.069
Femur ash weight (g)	0.36 (0.004)	0.38 (0.005)	0.011	0.37 (0.005)	0.37 (0.004)	0.749	0.38 (0.004)	0.37 (0.005)	0.112	0.37 (0.005)	0.37 (0.004)	0.801
Femur length (cm)	3.49 (0.03)	3.48 (0.03)	0.725	3.50 (0.03)	3.47 (0.03)	0.122	3.50 (0.03)	3.46 (0.03)	0.016	3.49 (0.04)	3.48 (0.03)	0.188
Femur Ca content (mg/g dry femur)	231.10 (2.70)	233.72 (2.47)	0.655	223.50 (2.82)	239.92 (2.05)	0.000	232.19 (2.08)	232.61 (3.05)	0.580	224.47 (2.99)	239.28 (1.92)	0.000
Femur Ca content (mg/g ash)	353.38 (3.79)	355.10 (3.58)	0.903	341.10 (3.77)	365.00 (3.13)	0.000	355.93 (2.83)	352.50 (4.39)	0.775	346.11 (4.26)	361.27 (2.96)	0.004
Femur Mg content (mg/g dry femur)	4.52 (0.08)	4.71 (0.08)	0.081	4.71 (0.10)	4.54 (0.06)	0.149	4.34 (0.06)	4.90 (0.08)	0.000	4.44 (0.09)	4.76 (0.06)	0.004
Femur Mg content (mg/g ash)	6.86 (0.12)	7.19 (0.12)	0.034	7.23 (0.15)	6.85 (0.09)	0.026	6.63 (0.10)	7.43 (0.12)	0.000	6.85 (0.14)	7.17 (0.10)	0.064
Femur P content (mg/g dry femur)	107.89 (2.89)	107.77 (1.93)	0.773	112.61 (3.01)	103.73 (1.79)	0.001	111.45 (1.89)	103.95 (2.88)	0.003	110.93 (3.02)	105.09 (1.82)	0.008
Femur P content (mg/g ash)	165.27 (4.51)	163.36 (3.10)	0.963	171.95 (4.82)	157.76 (2.73)	0.012	170.16 (2.92)	158.04 (4.59)	0.028	171.22 (4.73)	158.22 (2.83)	0.017
Femur Zn content (µg/g dry femur)	284.14 (4.56)	340.94 (10.29)	0.000	352.93 (10.45)	277.47 (3.63)	0.000	320.97 (8.67)	303.50 (8.39)	0.147	311.80 (10.56)	312.81 (6.65)	0.485
Femur Zn content (µg/g ash)	434.65 (6.98)	517.66 (16.61)	0.000	538.42 (16.66)	422.14 (5.79)	0.000	489.70 (13.47)	461.46 (13.51)	0.141	481.93 (17.07)	470.52 (10.00)	0.164

Values expressed as mean (standard error of the mean).

Table 10. Effects of the protein diet concentration, source of protein, resistance training and anabolic-androgenic steroids (AAS) on different bone parameters in femur (metaphysis and diaphysis).

	Protein diet concentration			Source of protein			Exercise			AAS		
Metaphysis	Normal protein (10%)	High protein (45%)	P	Animal (whey)	Vegetal (soy)	P	Sedentary	Resistance training	P	No AAS	AAS	P
Total BMD Metaphysis (mg/cm^3)	602.34 (5.84)	596.31 (5.09)	0.369	592.67 (5.72)	604.99 (5.23)	0.114	589.60 (5.26)	609.89 (5.49)	0.008	593.95 (5.23)	603.92 (5.60)	0.101
Cortical/Subcortical BMD (mg/cm^3)	1063.31 (4.55)	1066.97 (4.14)	0.553	1066.24 (4.06)	1064.16 (4.53)	0.852	1050.80 (4.14)	1080.44 (3.81)	0.000	1062.76 (4.30)	1067.05 (4.37)	0.341
Trabecular BMD (mg/cm^3)	250.35 (8.07)	249.33 (6.76)	0.885	244.03 (6.96)	254.69 (7.72)	0.268	256.12 (7.37)	243.12 (7.49)	0.219	242.86 (7.83)	255.67 (7.10)	0.208
Total cross-sectional area (mm^2)	15.58 (0.17)	16.30 (0.24)	0.005	15.96 (0.23)	15.91 (0.19)	0.492	16.48 (0.19)	15.35 (0.21)	0.000	16.39 (0.23)	15.55 (0.18)	0.005
Trabecular area (mm^2)	9.09 (0.15)	9.68 (0.19)	0.006	9.48 (0.19)	9.30 (0.16)	0.228	9.84 (0.15)	8.89 (0.17)	0.000	9.67 (0.19)	9.14 (0.15)	0.029
Cortical/Subcortical area (mm^2)	6.49 (0.07)	6.62 (0.07)	0.176	6.49 (0.07)	6.61 (0.07)	0.394	6.64 (0.07)	6.46 (0.07)	0.089	6.72 (0.08)	6.42 (0.06)	0.002
Diaphysis												
Total BMD Diaphysis (mg/cm^3)	902.54 (6.19)	898.98 (5.99)	0.662	899.70 (6.24)	901.71 (5.96)	0.736	905.27 (5.00)	896.00 (7.11)	0.288	895.35 (5.58)	905.34 (6.35)	0.256
Total cross-sectional area (mm^2)	9.50 (0.09)	9.95 (0.11)	0.002	9.61 (0.11)	9.82 (0.10)	0.221	9.83 (0.09)	9.61 (0.12)	0.110	9.81 (0.13)	9.65 (0.08)	0.279
Cortical BMD (mg/cm^3)	1388.70 (1.57)	1389.50 (1.58)	0.757	1387.51 (1.57)	1390.40 (1.55)	0.179	1388.37 (1.53)	1389.85 (1.62)	0.477	1388.49 (1.40)	1389.59 (1.67)	0.517
Cortical area (mm^2)	5.88 (0.05)	6.09 (0.06)	0.013	5.88 (0.06)	6.07 (0.05)	0.028	6.08 (0.05)	5.88 (0.06)	0.016	6.02 (0.07)	5.95 (0.05)	0.440
Cortical thickness (mm)	0.667 (0.005)	0.671 (0.004)	0.484	0.660 (0.004)	0.676 (0.004)	0.014	0.677 (0.004)	0.660 (0.005)	0.011	0.67 (0.005)	0.67 (0.004)	0.926
Periosteal perimeter (mm)	10.92 (0.05)	11.17 (0.06)	0.002	10.98 (0.06)	11.10 (0.05)	0.213	11.11 (0.05)	10.97 (0.7)	0.115	11.09 (0.07)	11.01 (0.05)	0.318
Endocortical perimeter (mm)	6.73 (0.05)	6.95 (0.05)	0.004	6.83 (0.06)	6.85 (0.05)	0.951	6.85 (0.04)	6.82 (0.06)	0.657	6.88 (0.07)	6.81 (0.05)	0.315

Values expressed as mean (standard error of the mean). BMD, bone mineral density.

Table 11. Effects of the protein diet concentration, source of protein, resistance training and anabolic-androgenic steroids (AAS) on plasmatic parameters and hormone levels in serum.

	Protein diet concentration			Source of protein			Exercise			AAS		
	Normal protein (10%)	High protein (45%)	P	Animal (whey)	Vegetal (soy)	P	Sedentary	Resistance training	P	No AAS	AAS	P
Serum Calcium (mg/dL)	11.95(0.53)	10.95(0.72)	0.262	9.15(0.62)	13.55 (0.54)	0.000	12.31(0.66)	10.53(0.58)	0.046	10.29(0.66)	12.54(0.58)	0.011
Serum Magnesium (mg/dL)	2.33(0.17)	2.57(0.26)	0.460	2.25(0.27)	2.63(0.17)	0.232	2.70(0.27)	2.18(0.14)	0.096	2.11(0.15)	2.78(0.26)	0.029
Serum Phosphorus (mg/dL)	6.59(0.32)	6.66(0.25)	0.858	6.34 (0.22)	6.87 (0.33)	0.184	6.93(0.30)	6.29(0.26)	0.110	6.36(0.26)	6.87(0.30)	0.214
Alkaline phosphatase (UI/L)	98.15(8.26)	102.49 (7.32)	0.695	101.00 (6.79)	99.25 (9.43)	0.881	82.17(6.16)	113.76 (7.75)	0.002	74.46(4.52)	148.16(5.55)	0.000
Urea (mg/dL)	25.56(0.70)	37.11(1.46)	0.000	31.91 (1.70)	30.29 (0.89)	0.399	29.88(1.59)	32.21(0.95)	0.210	31.87(1.44)	30.26(1.17)	0.303
Serum total proteins (g/dL)	5.54(0.07)	5.35(0.06)	0.048	5.60(0.06)	5.31(0.07)	0.002	5.35(0.07)	5.55(0.05)	0.037	5.53(0.08)	5.37(0.05)	0.084
Corticosterone (ng/mL)	867.11(46.61)	857.06 (31.31)	0.859	--	--	--	806.08 (34.1)	970.6 (35.30)	0.004	941.88(26.81)	693.32 (43.19)	0.000
Testosterone (ng/mL)	1.73(0.23)	2.83(0.37)	0.016	--	--	--	2.72 (0.32)	1.45(0.20)	0.001	2.15(0.23)	2.62(0.55)	0.055
Corticosterone/Testosterone ratio	6.58(0.87)	4.81(0.96)	0.178	--	--	--	4.40(0.61)	8.12(1.37)	0.006	5.65(0.79)	5.65(1.19)	0.999
Testosterone/Corticosterone ratio	0.23(0.02)	0.46(0.08)	0.006	--	--	--	0.43(0.06)	0.20(0.03)	0.002	0.30(0.04)	0.45(0.10)	0.201

Values expressed as mean (standard error of the mean).

Table 12. Effects of the protein diet concentration, source of protein, resistance training and anabolic-androgenic steroids (AAS) on some urinary parameters.

	Protein diet concentration			Source of protein			Exercise			AAS		
	Normal protein (10%)	High protein (45%)	P	Animal (whey)	Vegetal (soy)	P	Sedentary	Resistance training	P	No AAS	AAS	P
Urinary Calcium (mg/L)	2.04 (0.09)	3.41 (0.31)	0.000	3.35 (0.32)	2.16 (0.08)	0.002	2.27 (0.18)	2.29 (0.18)	0.914	3.39 (0.92)	2.13 (0.25)	0.000
Urinary calcium (mg/d)	0.51 (0.08)	0.80 (0.17)	0.130	0.77 (0.09)	0.67 (0.08)	0.426	0.51 (0.08)	0.96 (0.21)	0.064	1.02 (0.14)	0.55 (0.05)	0.003
Urinary Citrate (g/L)	1.96 (0.06)	0.71 (0.10)	0.000	0.85 (0.12)	1.80 (0.17)	0.000	1.62 (0.55)	1.12 (0.33)	0.040	1.26 (0.47)	1.49 (0.47)	0.351
Urinary pH	6.81 (0.07)	6.27 (0.03)	0.000	6.46 (0.05)	6.71 (0.06)	0.004	7.25 (0.17)	6.84 (0.15)	0.084	6.49 (0.17)	6.58 (0.15)	0.250
Kidney (g) (mean right and left)	0.96(0.02)	1.15(0.02)	0.000	1.05(0.02)	1.06(0.02)	0.766	1.11(0.02)	1.00(0.02)	0.000	1.05(0.02)	1.06(0.01)	0.505
Kidney (g/100g body weight)	0.30 (0.004)	0.36 (0.004)	0.000	0.32(0.01)	0.33(0.01)	0.404	0.33 (0.006)	0.32 (0.004)	0.579	0.31 (0.006)	0.34 (0.004)	0.001
Kidney (g/100g carcass)	0.57 (0.006)	0.67 (0.007)	0.000	0.63(0.01)	0.61(0.01)	0.202	0.63(0.01)	0.61(0.01)	0.150	0.61(0.01)	0.62(0.01)	0.629

Values expressed as mean (standard error of the mean).

Table 13. Effects of the protein diet concentration, source of protein, resistance training and anabolic-androgenic steroids (AAS) on blood parameters.

	Protein diet concentration			Source of protein			Exercise			AAS		
	Normal protein (10%)	High protein (45%)	P	Animal (whey)	Vegetal (soy)	P	Sedentary	Resistance training	P	No AAS	AAS	P
White blood cell count (/µL)	6241.51 (389.63)	5123.08 (355.47)	0.037	6298.28 (332.28)	4934.04 (415.36)	0.011	5366.07 (350.31)	6055.10 (410.31)	0.202	6000.00 (346.99)	5254.55 (418.55)	0.172
Red blood cells count (/µL)	8593065 (107086.95)	8602712 (113550.59)	0.951	8631940 (98567.36)	8555370 (124495.27)	0.626	8487656 (124081.69)	8721404 (86295.75)	0.133	8468676 (81006.86)	8763396 (141282.21)	0.074
Haemoglobin (g/dL)	15.19(0.16)	15.36(0.20)	0.508	15.21(0.17)	15.37(0.20)	0.533	15.09(0.20)	15.48(0.15)	0.124	15.09(0.14)	15.53(0.22)	0.084
Serum iron (µg/dL)	164.92(6.21)	160.71(6.45)	0.639	156.46(5.54)	158.50(6.85)	0.172	176.00(5.46)	149.69(6.74)	0.003	159.60(7.03)	165.90(5.62)	0.482
Haematocrit (%)	46.42(0.56)	46.51(0.60)	0.912	46.27(0.51)	46.70(0.67)	0.607	45.70(0.66)	47.32(0.44)	0.043	46.08(0.43)	46.94(0.75)	0.321
Mean corpuscular volume (fL)	54.05(0.18)	54.15(0.20)	0.726	53.64(0.15)	54.66(0.21)	0.000	53.89(0.18)	54.32(0.20)	0.108	54.49(0.18)	53.60(0.18)	0.001
Mean corpuscular haemoglobin (pg)	17.46(0.09)	17.83(0.14)	0.035	17.63(0.12)	17.65(0.13)	0.935	17.69(0.13)	17.58(0.11)	0.479	17.71(0.10)	17.55(0.15)	0.342
Mean corpuscular haemoglobin concentration (g/dL)	32.32(0.17)	32.93(0.23)	0.035	32.88(0.20)	32.29(0.20)	0.041	32.87(0.24)	32.34(0.13)	0.067	32.50(0.12)	32.77(0.29)	0.407
Platelet (µL)	352844.8 (41825.02)	347736.8 (43403.80)	0.933	360508.2 (42458.18)	338796.3 (42568.01)	0.720	290983.3 (35167.58)	415036.4 (48470.53)	0.041	313123.1 (34860.77)	398660.0 (51646.80)	0.158
Lymphocytes (%)	65.76(1.19)	67.88(1.21)	0.215	67.67(1.18)	65.67(1.22)	0.247	65.44(1.23)	68.31(1.14)	0.094	67.74(0.82)	65.41(1.71)	0.182

Values expressed as mean (standard error of the mean).