Revista de Osteoporosis y Metabolismo Mineral

2º Curso de Osteoporosis y Enfermedades Metabólicas Óseas para Médicos Residentes

MADRID, JUEVES 15 DE JUNIO 2017 *

HOSPITAL UNIVERSITARIO INFANTA LEONOR
Gran Vía del Este, 80 - 28031 Madrid

Introducción y formación básica en el área de las enfermedades metabólicas óseas. Tanto la osteoporosis como otras enfermedades metabólicas óseas requieren de un manejo multidisciplinar con lo que este curso tiene como objetivo dar a conocer aspectos básicos de la epidemiología, diagnostico, manejo clínico y farmacológico de estas enfermedades

PROGRAMA

PRESENTACIÓN DE LA JORNADA
9-9:15 Bienvenida/Entrega de la documentación
9:15-9:30 Inauguración del curso

BLOQUE 1: FISIOPATOLOGIA Y EPIDEMIOLOGÍA DE LA OSTEOPOROSIS
9:30-10 Nuevos datos de la fisiopatología de la osteoporosis (el remodelado óseo a nivel celular y molecular. El sistema rankl. La vía Wnt-catenina)
Dr. Guillermo Martínez Díaz-Guerra
10-10:30 Epidemiología de la osteoporosis en España
Dr. Ricardo Larrainzar

BLOQUE 2: FACTORES DE RIESGO Y DIAGNÓSTICO DE LA OSTEOPOROSIS
10:30-11 Pruebas complementarias en el abordaje inicial. (Escalas de riesgo. Cuándo solicitar y cómo interpretar una densitometría. Analítica para estudio de secundarias. Marcadores remodelado óseo. Otras exploraciones)
Dra. Mª Jesús Moro

11-11:30 CAFÉ

BLOQUE 3: ENFERMEDADES METABÓLICAS ÓSEAS DE MAYOR PREVALENCIA
11:30-12 Osteoporosis (postmenopáusica, del varón, corticoidea osteoporosis secundaria)
Dr. José Luis Pérez Castrillón
12.-12:30 Enfermedad de Paget
Dr. Javier del Pino
12:30-13 Efectos sobre el metabolismo óseo de la cirugía bariátrica. Osteomalacia. Osteodistrofia renal
Dr. José Luis Pérez Castrillón

13-13:30 Hiperparatiroidismo primario, hiperparatiroidismo normocalcémico, hipoparatiroidismo postquirúrgico e idiopático
Dr. Guillermo Martínez Díaz-Guerra

13:30-15 COMIDA

BLOQUE 4: FORMAS DE PRESENTACIÓN CLÍNICA DE LA OSTEOPOROSIS FRACTURA OSTEOPORÓTICA
15-15.30 Fractura no vertebral (humero, radio y cadera)
Dr. Ricardo Larrainzar
15.30-16 Fractura vertebral
Dra. Elena Martínez

BLOQUE 5: TRATAMIENTO NO FARMACOLOGICO Y FAMACOLÓGICO DE LA OSTEOPOROSIS. GUÍA CLINICA DE LA SEIOMM
16-16:30 Tratamiento de la osteoporosis. Guia clínica SEIOMM
Dr. Javier del Pino
16.30-17 Efectos adversos de los tratamientos
Dra. Elena Martínez
17-17.30 Tratamiento del dolor en la osteoporosis (analgésicos, vertebroplastia y cifoplastia, bloqueos anestésicos)
Dr. Alejandro Ortega

17.30-18 CAFÉ

18-19 Osteotrivial: taller casos clínicos.
Dra. Mª Jesús Moro

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SUMMARY

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Divergent effects of vascular endothelial growth factor, VEGF and the N-terminal fragment of the parathormone-related protein, PTHrP on human adipose derived from mesenchymal stem cells

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The possibility of obtaining stem cells from adult tissues is highly attractive as they can potentially generate a variety of differentiated cells and be used in tissue regeneration. The study of stem cells obtained from adult organisms began some 50 years ago, when hematopoietic stem cells were described, which give rise to all blood cells[1]. Later, researchers described mesenchymal lineage stem cells and differentiated them into adipocyte, osteoblastic and chondrocytic cells. Mesenchymal stem cells were originally discovered in bone marrow, but were later found in other adult tissues, including peripheral adipose tissue. As Bravo et al.[2] report in their study, mesenchymal cells are characterized by the expression of surface markers, including CD90, and the use of others, such as CD45 and CD34.

The advantages of adipose tissue as a source of stem cells are its abundance in adults and the fact that it can be obtained by minimally invasive procedures such as liposuction. Once adipose tissue is obtained and treated enzymatically to remove extracellular proteins, stem cells can be separated from mature adipocytes by centrifugation, taking advantage of the low density of the adipocytes floating in the isolation medium. The cells at the bottom of the centrifuge tube, called the vascular stromal fraction, contain the so-called ASC (adipocyte stem cell or adipocyte stem cells). In the appropriate medium, ASCs can be differentiated into adipocytes, osteoblasts/osteocytes and chondrocytes or even into glial and neuronal cells[1].

The beneficial effect of parathyroid hormone (PTH) on the bone is widely recognized[3,4]. When the hormone is administered intermittently, it can activate the parathormone 1 receptor (PTH1R), triggering an increase in the number of osteoblasts, which leads to increased bone formation and bone mass. Its administration in humans is the only anabolic treatment approved by the Food and Drug Administration (FDA). Parathyroid hormone-related protein (PTHrP) is an analog of PTH capable of activating PTH1R through its N-terminal region[5]. Similar to PTH, clinical studies have shown that fragments containing the N-terminal region of PTHrP increase bone mass in postmenopausal women with osteoporosis.

Vascular endothelial growth factor (VEGF) is a cytokine produced by cells that are part of, or directly associated with, blood vessels[6]. VEGF is also produced by osteoblasts and participates in the development of endochondral, intramembranous bone and bone repair.

The research group that carried out this study[2] previously demonstrated the role of receptors for PTH and VEGF in the response of osteocytes to mechanical impulses[7,8]. These studies established the involvement of PTH1R and VEGF receptor 2 in the anti-apoptotic effect of mechanical stimulation exerted by fluid flow. In the present study by Bravo et al.[2] ASCs are shown to respond differently to VEGF and PTHrP (1-36). Treatment with pro-differentiating media leads to the production of alkaline phosphatase and mineral accumulation, along with the expression of osteoprotegerin and Runx2 in the ASCs. In contrast to the similar effect that receptors for PTH and VEGF exert on the viability of osteocytes subjected to mechanical stimulation, PTHrP (1-36) and VEGF do not have the same effect on the proliferation of ASCs. In particular, VEGF stimulates the proliferation of undifferentiated cells, whereas PTHrP (1-36) has no effect on growth medium. On the other hand, PTHrP (1-36), but not VEGF, stimulates the proliferation of ASCs maintained in medium supplemented with ascorbic acid and β-glycerophosphate to induce differentiation of cells into the osteoblastic lineage. The authors suggest that VEGF would be more effective in increasing the number of cells that remain undifferentiated in the vicinity of blood vessels, particularly in the presence of endothelial cells. On the other hand, PTHrP stimulates the proliferation of cells involved in the osteo-

blastic lineage in the proximity of more mature cells. These studies suggest the possibility of treatments combining the 2 agents to increase the amount of cells in cultures of ASCs that can be used to promote bone regeneration, for example, in individuals with fractures that cannot spontaneously weld.

Bibliography

1. Lo Furno D, Mannino G, Cardile V, Parenti R, Giuffrida R. Potential therapeutic applications of adipose-derived mesenchymal stem cells. Stem Cells Dev. 2016 Sep 22. [Epub ahead of print].
2. Bravo B, Fernández de Castro L, Buendía I, Santos X, Gortázar A. El factor de crecimiento endotelial vascular (VEGF) y el fragmento N-terminal de la proteína relacionada con la parathormona (PTHrP) regulan la proliferación de células madres mesenquimales humanas. Rev Osteoporos Metab Miner. 2017;9(1):5-12.
3. Jilka RL. Molecular and cellular mechanisms of the anabolic effect of intermittent PTH. Bone. 2007;40:1434-46.
4. Bellido T, Saini V, Divieti Pajevic P. Effects of PTH on osteocyte function. Bone. 2013;54:250-7.
5. Esbrit P, Herrera S, Portal-Nunez S, Nogues X, Diez-Perez A. Parathyroid Hormone-Related Protein Analogs as Osteoporosis Therapies. Calcif Tissue Int. 2016;98:359-69.
6. Hu K, Olsen BR. Vascular endothelial growth factor control mechanisms in skeletal growth and repair. Dev Dyn. 2016 Oct 17.[Epub ahead of print].
7. de Castro LF, Maycas M, Bravo B, Esbrit P, Gortazar A. VEGF Receptor 2 (VEGFR2) activation is essential for osteocyte survival induced by mechanotransduction. J Cell Physiol. 2015;230:278-85.
8. Maycas M, Ardura JA, de Castro LF, Bravo B, Gortazar AR, Esbrit P. Role of the parathyroid hormone type 1 receptor (PTH1R) as a mechanosensor in osteocyte survival. J Bone Miner Res. 2015;30:1231-44.

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Vascular endothelial growth factor (VEGF) and the N-terminal portion of parathyroid hormone-related protein (PTHrP) regulate the proliferation of human mesenchymal stem cells

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Summary

Adipose tissue contains a large number of mesenchymal stem cells (ASCs) residing in their vascular stroma. Although there is controversy regarding the ability to generate bone tissue from these cells *in vivo*, the *in vitro* cells offer a good model of osteogenic differentiation due to its phenotypic similarity with the bone marrow stromal cells (BMSCs) in culture. The differentiation of osteo-progenitor populations of bone marrow is intensely regulated by local factors, such as vascular endothelial growth factor (VEGF) and parathyroid hormone-related protein (PTHrP), which modulate these populations' proliferation in different stages of differentiation. Both the VEGF and the N-terminal fragment of the PTHrP exert osteogenic effects. In this study, we posited that its effects on proliferation of osteo-progenitors are stage dependent of osteoblastic differentiation. After confirming its capacity to *in vitro* differentiation by Runx2 gene expression and accumulation of calcium, the proliferative response to stimuli was analyzed with VEGF or PTHrP (1-36) of ASCs submitted or not to osteogenic induction. VEGF, but not PTHrP (1-36), stimulated the proliferative capacity of uninduced ASCs, whereas BMSCs, but not VEGF, stimulated the proliferation of induced ASCs, corroborating the differential role of this growth in different stages of differentiation.

Key words: *adipose mesenchymal stem cells (ASCs), PTHrP, VEGF, osteogenic differentiation.*

Introduction

In the late 1960s, Friedenstein first described Bone Marrow Stromal Cells (BMSCs) as fibroblastic cells with adhesion to the plastic and tri-linear differentiation capacity, generating osteoblasts, chondroblasts and adipocytes. A fraction of these cells also demonstrated clonogenic capacity when they were cultured in very low density (Colony Forming Units-Fibroblast, CFU-F)[1]. It was later noted that this multipotentiality is only inherent in a small part of this heterogeneous cell population, corresponding to Skeletal Stem Cells (SSCs, less than 0.1% of BMSCs and about 12% of CFU-F), which are also the only ones able to produce the stromal necessary for generating hematopoietic niches in bone marrow[2,3].

Adipose tissue contains a large number of adherent cells, capable of forming CFU-F and many other features that liken them to BMSCs. Compared to BMSCs, this tissue is easily accessible, such as liposuction discarded in liposuction operations, and isolation of stromal cells is relatively simple[4,5]. Similar to the SSCs among BMSCs, the stromal vascular fraction (SVF) of adipose tissue is a heterogeneous population of cells, that include the ASCs, which presumably are similarly associated with SSCs to the microvasculature of fat[6], in consonance with other tissues in which the adult stem cells are associated with the microvasculature in the form of pericytes[7]. Although several authors claim that ASCs have the inherent ability to differentiate into bone-derived cells, to date no research group has demonstrated that this is possible except after trans-differentiating these cells after intensive treatment with BMPs, which has been shown to be a potent osteo-inductor of various cell types[8]. Although the clinical utility of ASCs for bone regeneration has not been demonstrated, these cells may be a convenient model for studying osteoblastic differentiation *in vitro*, because of its easy access and response similar to that of BMSCs to *in vitro* tri-linear differentiation factors[9].

In the presence of ascorbic acid, dexamethasone and β-glycerol-phosphate the ASCs express markers of osteoblastic differentiation *in vitro*. This is the case of the system consisting of osteoprotegerin (OPG) and activator receptor ligand for the nuclear factor kB (RANKL), proteins involved in bone remodeling. RANKL is a protein of the tumor necrosis factor α (TNF-α) family that is expressed on the membrane of osteoblasts, which in turn can be secreted by them[10]. It binds to the activator receptor for the nuclear factor kB (RANK) that is present in osteoclast precursors activating its differentiation and maturation to osteoclasts[11]. OPG is a decoy receptor secreted by osteoblasts that binds to RANKL, preventing it from binding to RANK, thereby blocking the activation of osteoclasts. On the other hand, Runx2 is a key transcription factor in the differentiation of osteoblastic cells[12].

Vascular endothelial growth factor (VEGF) is a key molecule in the regulation of endothelial cell proliferation. It promotes the proliferation, migra-tion and survival of these cells, as well as their vascular permeability[13]. The expression of VEGF and its receptors in BMSCs has been demonstrated by various studies in cell cultures[13,14]. In addition, the role of the VEGFR2 receptor as key in osteoblastic differentiation and survival has been shown *in vitro*[15]. VEGF induces differentiation in cell cultures of preosteoblasts[14] and stimulates their migration and proliferation[13,14]. Thus, VEGF seems to be involved in the early stages of bone differentiation, both in skeletogenesis –an important factor in endochondral and intramembranous bone formation– and in adult homeostasis, promoting osteoblastic differentiation and reducing adipogenic differentiation of BMSCs[16,17].

Parathormone-related protein (PTH), PTHrP, is a pleiotropic cytokine with important functions in bone tissue[15]. It is considered a modulator of bone remodeling and a stimulator of bone formation that promotes osteoblastic differentiation and its survival[15]. It is, therefore, an important maturation factor. The post-translational processing of the PTHrP gene generates different fragments, including an N-terminal fragment, PTHrP (1-36), which shows great homology with PTH and acts through the common 1 receptor for PTH/PTHrP, PTH1R[12]. The expression of PTHrP in the osteoblastic lineage decreases as it differentiates[20], but PTH1R plays a fundamental role in mature osteoblasts and osteocytes, decreasing its apoptosis and increasing its number in periosteum osteoblasts *in vivo*[18,19]. Previous studies indicate that PTHrP could increase the proliferation of immature osteoblasts through the regulation of Cyclin D1 (promoter) and p27 (inhibitor), both regulators of the cell cycle[12].

In this paper, we hypothesize that the effects of VEGF and PTHrP on cell proliferation of osteoprogenitors are dependent on the osteoblastic differentiation stage. Thus, more undifferentiated populations would be more sensitive to VEGF while the progenitors already committed to osteoblastic differentiation would respond better to PTHrP (1-36). For this purpose, human ASCs from liposuction were used as an *in vitro* model of osteogenic differentiation. After confirming the model's *in vitro* differentiation ability by Runx2 gene expression and calcium accumulation, the proliferative response to stimuli with VEGF or PTHrP (1-36) of ASCs subjected to or not undergoing osteogenic induction was analyzed. VEGF, but not PTHrP (1-36), induced proliferative capacity of undifferentiated ASCs, whereas PTHrP (1-36), but not VEGF, induced proliferation of ASCs previously treated with osteogenic differentiation medium, confirming the differential role of these growth factors in different stages of differentiation.

Materials and methods
Isolation, primary culture and functional study of ASCs

Abdominal subcutaneous adipose tissue was obtained during the surgery of healthy patients using the liposuction technique. Six women were

included in this study with an average age of 40 years (range 25-60 years). All donors gave their informed consent, according to the appropriate clinical protocol. Patients were operated in the Department of Plastic Surgery of HM Hospitals (Madrid, Spain), and tissue sample collection was approved by the Institutional Review Board/Clinical Research Ethics Committee of HM Hospitals (Madrid, Spain). Adipose tissue was digested in 0.075% collagenase solution type I (Invitrogen, Life Technologies, New York, USA) for 30 minutes at 37°C, following the protocol described previously[4]. The cells were then plated on plastic surface (Corning, New York, USA) for 24 hours in DMAX Growth Medium (Invitrogen, Life Technologies, New York, USA) with 10% FBS (Sigma St. Louis, Missouri, USA) and supplemented with antibiotics: penicillin (100 IU/ml) and streptomycin (100 mg/ml) (Sigma-Aldrich, St. Louis, Missouri, USA). Non-adherent cells were removed, and fresh medium was added for the primary culture of the adherent cell fraction for 7 days. The culture medium was replaced every 3 days. At that time, ASCs were functionally phenotyped by flow cytometry and their potential for osteogenic differentiation was analyzed.

Flow cytometry
For flow cytometry analysis, ASCs were re-suspended in PBS (saline phosphate buffer) at a density of 1x10 6 cells/ml, fixed with 2% paraformaldehyde (PFA) and incubated with conjugated mouse monoclonal antibodies (FITC or PE) with anti-CD90, CD29, CD34, CD45, CD106 CD44, CD144, CD31 and KDR (BioLegend, San Diego, CA, USA) for 30 min at room temperature. Cells were washed 3 times with PBS and analyzed by Accuri's C6 Flow System cytometer at 488 nm and 15 mW. Frontal dispersion (FSC), lateral dispersion (SSC) and specific fluorescent marker (LGFL) at 488 nm and 540 nm were automatically obtained for each cell. Data were digitally collected over a dynamic range of 16 million digital data channels. Amplification and logarithmic analysis of fluorescence was performed using BD Accuri™ c6 Analysis Software.

Assays of osteogenic differentiation
Cell lineage osteogenic differentiation medium was used to evaluate the potential for osteogenic differentiation of ASC. For this differentiation, ASCs were cultured for 14 days in the presence of DMEM (Dulbecco's Modified Eagle medium) with 10% FBS, 10^{-8} M dexamethasone, 0.5 mg/ml ascorbic acid and 0.1 M β-glycerolphosphate Sigma-Aldrich, St. Louis, Missouri, USA). The medium was replaced every 3 days, and at the end of the 14-day period, the histochemical red alizarin staining was performed to reveal and quantify the number of cells per microscopic field surrounded by mineralized extracellular matrix stained with alizarin red. A check of osteoblastic differentiation was also performed by means of alkaline phosphatase (ALP) assay, which consisted of the iden-

tification of the red stained deposits indicating alkaline phosphatase activity by the Sigma Aldrich 86R kit. ASCs from six different patients were used in each test.

Cellular proliferation studies
The response related to the proliferation of ASCs to factors such as VEGF (160 pM) and PTHrP (1-36) (100 nM) in the cultures were studied for 24 hours in both growth medium and osteogenic differentiation medium. Measuring the proliferation of these assays, the xCelligent System (Roche Diagnostics, Basel, Switzerland) was used to measure cell proliferation in real time. The xCelligent system provided real-time and end-point proliferation measurements based on readings of cultured plates with electrodes that detected changes in cell morphology, providing a parameter called Cell Index extrapolable to cell proliferation.

Extraction of total RNA and quantitative PCR in real time
ASCs, both at baseline and at differentiated conditions to osteoblasts, were subjected to extraction of total RNA from a cell homogenate with a standard method with guanidyl-phenol-chloroform thiocyanate (Tri-Reagent©, MCR; Cincinnati, Ohio, USA). The purity and quantification of total RNA extracted was determined by spectrophotometry A_{260}/A_{280} (Nanodrop 2000/2000c Spectrophotometer, Thermo Scientific). Synthesis of cDNA was performed using random oligonucleotides and a reverse transcriptase (High capacity RNA to cDNA Kit, Applied Byosystem; Foster City, Calif., USA). Gene expression analysis by real-time RT-PCR was performed with resulting cDNA, using a heat-activated polymerase, TaqDNA (Taqman gene expression master mix, Applied Byosystem, Foster City, California, USA) and human-specific primers For RUNX2, (Hs00231692 m1) OPG (TNFRSF1 Hs 00171068), VEGF-A (Hs 00173626 m1) and VEGFR2 (Hs 00176676 m1) (Applied Byosystem; Foster City, California, USA). After an initial incubation of 10 minutes at 25°C and another of 2 hours at 37°C, the samples were cycled at 4°C. The results were expressed as expression levels of each gene (once normalized to the 18s RNA as constitutive gene) in each experimental condition, relative to its corresponding control: 2 -ΔCt, where Ct represents the PCR threshold cycle in the Which the program detects for the first time an appreciable increase of fluorescence on the basal signal. All determinations were performed in duplicate. (ΔCt = Ct (gene of interest) - Ct (18S endogenous control).

Statistical analysis
Results were expressed as mean +/- Standard Deviation (SD). The non-parametric comparison between the means of two samples was performed by the Mann Whitney test. Non-parametric ANOVA was used to compare several samples (Kruskal-Wallis). All values with p <0.05 were considered significant.

Results

Firstly, the characterization of ASCs from the primary culture of liposuction was carried out. Phenotypic analysis of ASC by flow cytometry revealed that 99.6% were CD90^{+}, 91,6% CD44^{+}, 90,4% CD 29^{+}, 4,2% CD34^{+}, 2,2% CD45^{+}. In addition, the markers CD106, CD155, KDR and CD31, all negative for ASCs (Table 1), were analyzed.

To evaluate the potential for osteogenic differentiation of ASC, they were subjected to osteoblastic differentiation *in vitro*. As shown in Figure 1A, ALP activity revealed that ASCs in osteogenic differentiation medium had undergone such differentiation. The red cytochemical staining of alizarin (Figure 1B) also revealed the positive result of osteogenic differentiation.

Gene expression was evaluated for some of the markers of osteogenic differentiation such as OPG and Runx2[12,13,24]. The expression of these two markers was significantly increased (p <0.05) after 14 days of culture with the osteogenic differentiation medium (Figure 2A-B).

The gene expression of the VEGFA system and its receptor induced by osteogenic differentiation was evaluated. As can be seen in Figure 3, after 7 days of differentiation the cells significantly decrease the expression of VEGF and its type 2 receptor.

Cell proliferation assays with media supplemented with PTHrP and VEGF showed different results. A proliferation study of the ASCs in response to VEGF (160 pM) was performed for 24 hours under basal conditions (growth medium) or with osteogenic differentiation medium.

As shown in Figure 4, VEGF significantly increased the proliferation of ASCs in growth medium, but had no significant effect on proliferation of cultured cells in osteogenic differentiation medium (p <0.05). The results indicated that VEGF increased the proliferation of ASCs, but provided they did not begin their differentiation process. In response to PTHrP (1-36) (100 nM) for 24 hours, ASCs did not significantly alter their proliferation index (Figure 4A). When the above-described proliferation assay was performed on osteogenic differentiation medium, it was found to change the ASC phenotype, and altered its responsiveness to these factors. Thus, in this case, PTHrP increased proliferation index in cells already committed to osteoblastic differentiation (Figure 4B).

Discussion

In the present study, ASCs from healthy women were exposed to short treatments with PTHrP and VEGF under different differentiation conditions and their proliferative capacity was analyzed. Our results indicate that the more undifferentiated populations would be more sensitive to VEGF while the progenitors already committed to osteoblastic differentiation respond better to PTHrP (1-36), showing a greater expression of PTH/PTHrR (PTH1R) receptor 1. Human ASCs derived from liposuction as an *in vitro* model of osteogenic differentiation were used. During this study the characterization of the ASCs was carried out by a study of the cell surface markers. The data obtained were those expected according to previous studies[21,22]. The capacity of osteoblastic differentiation of ASCs was also studied. Such differentiation was verified by the ALP activity of these cells after 14 days in osteogenic differentiation medium. Similarly, histochemical staining of alizarin red was used to verify such differentiation thereby complementing the analysis of ALP activity. In addition, the gene expression of some markers such as OPG and Runx2,[16,23] was evaluated. Runx2 is essential for osteoblastic differentiation, leading ASCs to the osteoblastic lineage and inhibiting their differentiation into adipogenic or chondrogenic lineage[23]. In addition, Runx2 has been described as a factor that keeps osteoblasts in the imma-

Table 1. Characterization by flow cytometry of ASCs isolated from adipose tissue obtained by liposuction

Surface marker	Expected expression	% mean expression obtained
CD 90	(+) for mesenchymal cell	94.6%
CD 44	(+) for mesenchymal cell	91.6%
CD 29	(+) for mesenchymal cell	90.4%
CD 45	(-) for mesenchymal cell	2.2%
CD 34	(-) for mesenchymal cell	4.2%
CD 106	(+) for endothelial cells	7.6%
	(-) for mesenchymal cell	
CD 144	(+) for endothelial cells	0.6%
KDR	(+) for endothelial cells	0.5%
CD 31	(+) for endothelial cells	0.2%

ture state without differentiating into osteocytes[23]. Likewise, previous studies consider OPG as a marker of early osteogenic differentiation in human mesenchymal cells[24]. As expected, our data show that osteogenic differentiation induces an increase in the expression of these two markers, OPG and Runx2, with respect to the undifferentiated state.

Gene expression of the VEGFA system and its VEGFR2 receptor were also evaluated under conditions of osteogenic differentiation. After seven days in the presence of the osteogenic differentiation medium, a decrease in the expression of VEGF and its type 2 receptor was observed. In our study, we hypothesized that the effects of VEGF and PTHrP on cell proliferation of osteoprogenitors are dependent on the stage of differentiation Osteoblast. Thus, the more undifferentiated populations would be more sensitive to VEGF due to their proximity to the endothelial niche *in vivo*, whereas the already compromised progenitors towards osteoblastic differentiation would respond better to PTHrP (1-36) by showing the PTH1R receptor a more important role in osteoblasts Mature and osteocytes. After confirming the model's *in vitro* differentiation ability by Runx2 gene expression and mineralization, the pro-

liferative response to VEGF or PTHrP (1-36) stimuli of ASCs subjected to or not undergoing osteogenic induction was analyzed. VEGF, but not PTHrP (1-36), favored the proliferative capacity of uninduced ASCs, whereas PTHrP (1-36), but not VEGF, favored the proliferation of previously differentiated ASCs, confirming the differential role of these growth factors in different stages of differentiation. Previous studies indicate that secreted VEGF is critical in the differentiation of BMSCs into osteoblasts, hindering their differentiation to other cell lineages such as adipogenic[16,17]. Likewise, Alonso et al.[15] have shown how VEGF and PTHrP modulate the differentiation and survival of osteoblasts. Our results from the proliferation study of ASCs in response to VEGF for 24 hours in growth medium or with osteogenic differentiation medium show an increase in the proliferation of ASCs grown in normal medium but without significant effects on cell proliferation cultured in the middle of osteogenic differentiation, supporting the notion that VEGF exerts a preponderant role on the regulation of proliferation in early stages of differentiation, although previous studies also point to an implication of VEGF in the survival of osteoblasts[15].

Figure 1. Primary ASC culture response to specific means of cell line differentiation. (A) Detection of alkaline phosphatase activity (ALP) and (B) detection of 14-day osteogenic differentiation by alizarin red staining to detect mineralization
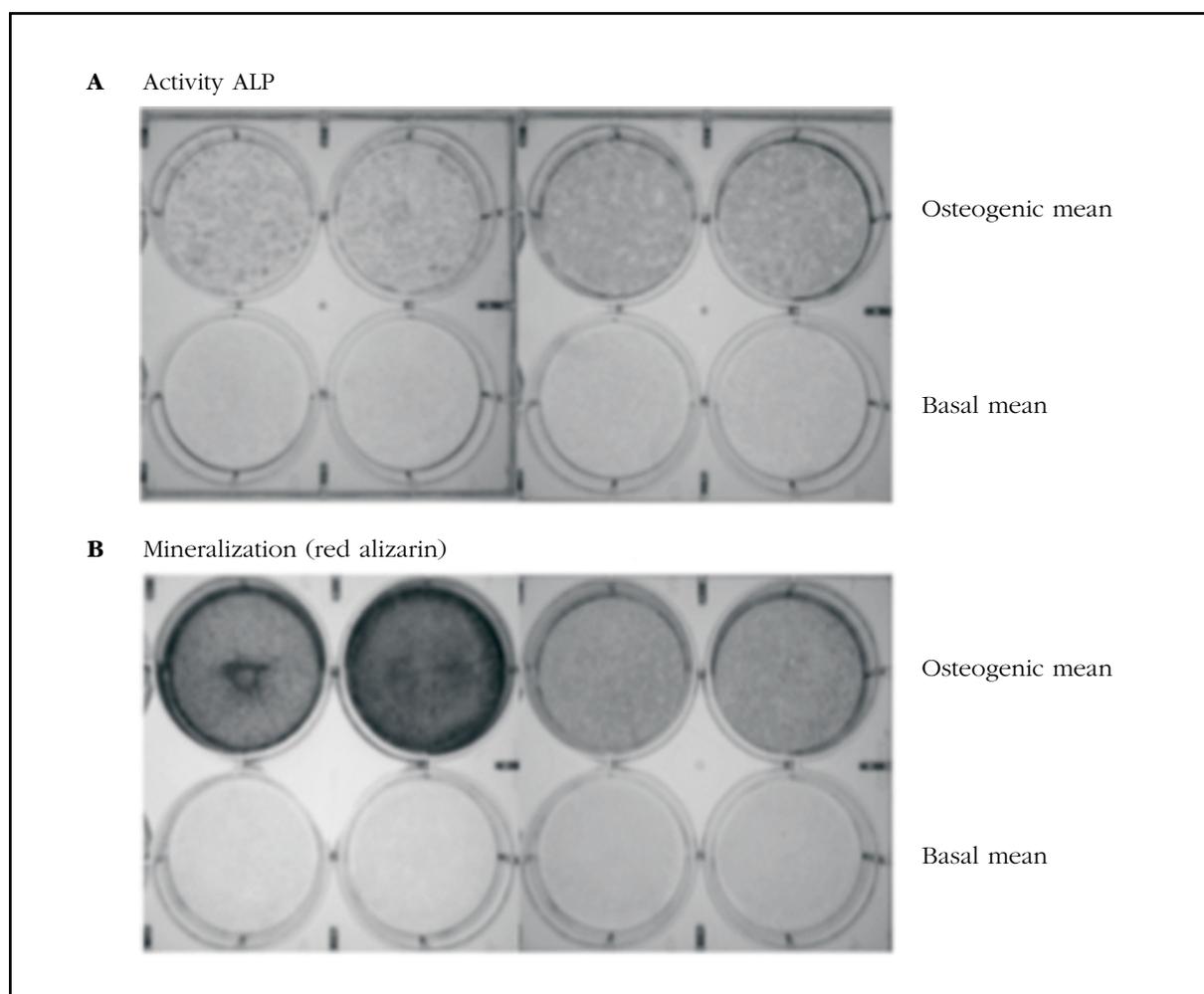
Figure 2. Changes in gene expression (analyzed by real-time PCR) of bone differentiation factors: (A) OPG, (B) Runx2, at different times of osteoblastic differentiation
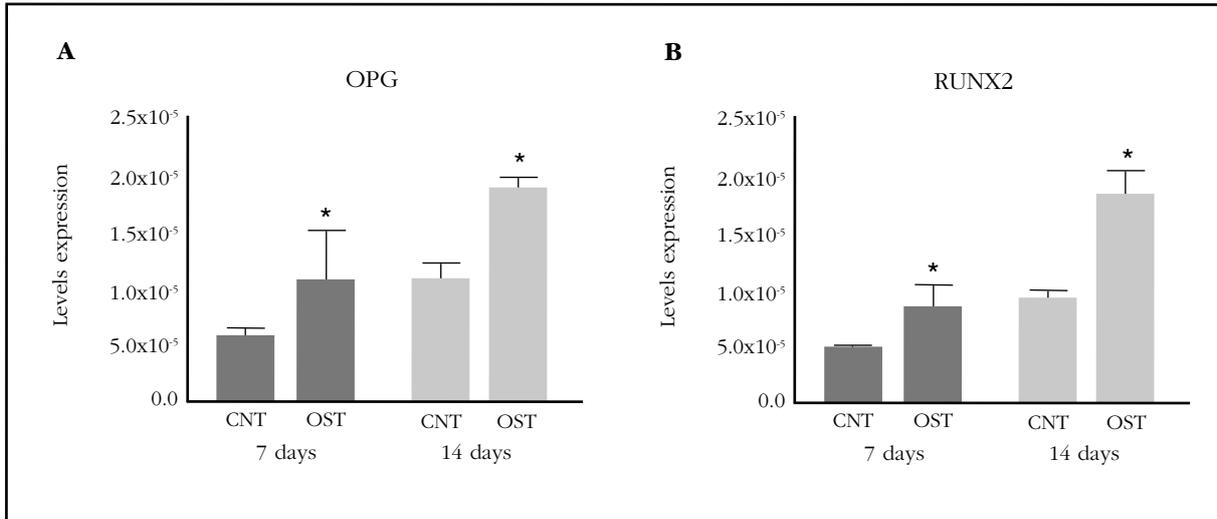
(*) p<0.05 *vs.* corresponding basal value.

Figure 3. Changes in gene expression after 7 days of differentiation (analyzed by real-time PCR) of angiogenesis factors: (A) VEGF-A and (B) VEGFR2
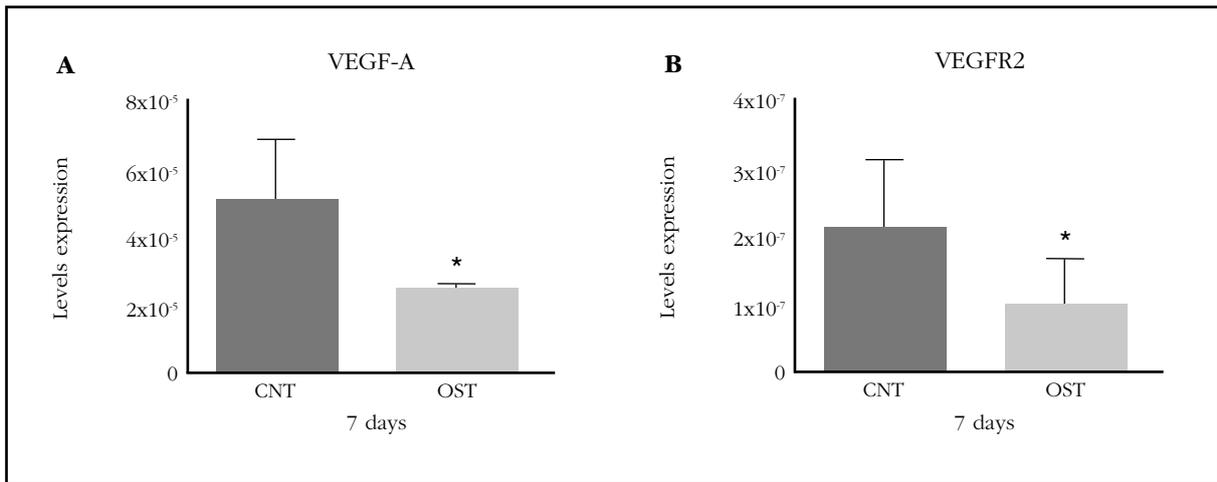
(*) p<0.05 *vs.* corresponding basal value.

Figure 4. Cell proliferation index (103) in ASC cultures undergoing stimuli with 160 pM VEGF165 and 100 nM PTHrP for 24 hours. (A) Proliferation in growth medium. (B) Proliferation in the middle of osteogenic differentiation
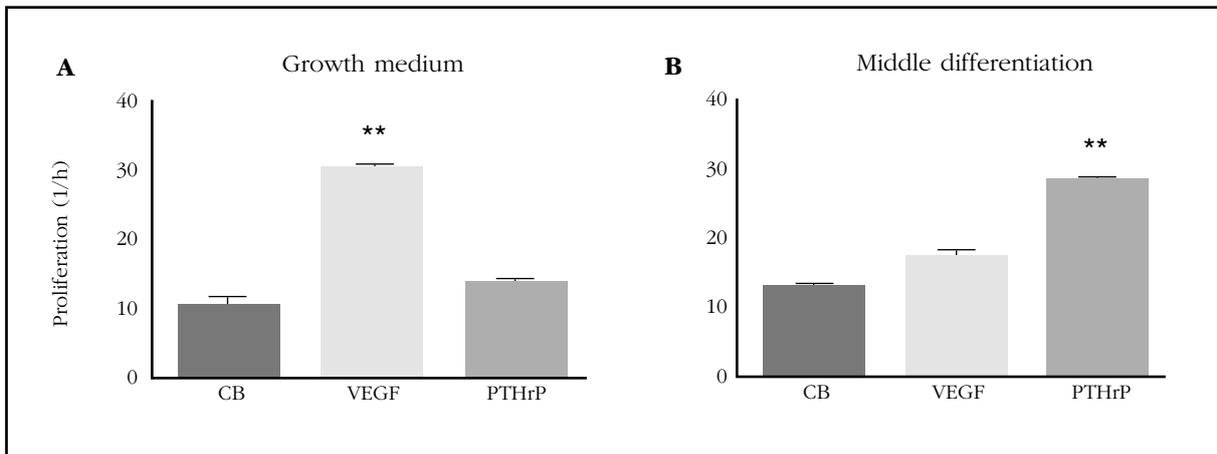
(**) p<0.05 *vs.* corresponding basal value.

After 24 hours of treatment with PTHrP (1-36), undifferentiated ASCs did not alter their proliferation index. However, in the presence of osteogenic differentiation medium, PTHrP (1-36) significantly increased proliferation, pointing to the implication of this factor in the proliferation of BMSCs involved in osteogenic differentiation *in vivo*.

Thus, we may conclude that, although previous studies have shown that VEGF and PTHrP modulate the differentiation and survival of osteoblasts[15], these factors could regulate the proliferation of osteoprogenitors depending on their commitment or not to the osteoblastic differentiation, with VEGF more involved in the proliferation of more undifferentiated progenitors close to the perivascular niche, whereas PTHrP (1-36) would stimulate cells more involved in osteoblastogenesis.

Conflict of interest: The authors declare they have no conflict of interest regarding this work.

Bibliography

1. Friedenstein AY, Lalykina KS. Lymphoid cell populations are competent systems for induced osteogenesis. Calcif Tissue Res. 1970;Suppl:105-6.
2. Bianco P, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell. 2008;2(4):313-9.
3. Sworder BJ, Yoshizawa S, Mishra PJ, Cherman N, Kuznetsov SA, Merlino G, et al. Molecular profile of clonal strains of human skeletal stem/progenitor cells with different potencies. Stem Cell Res. 2015;14:297-306.
4. Van Harmelen V, Röhrig K, Hauner H. Comparison of proliferation and differentiation capacity of human adipocyte precursor cells from the omental and subcutaneous adipose tissue depot of obese subjects. Metabolism. 2004;53:632-7.
5. Prunet-Marcassus B, Cousin B, Caton D, André M, Pénicaud L, Casteilla L. From heterogeneity to plasticity in adipose tissues: site-specific differences. Exp Cell Res. 2006;312:727-36.
6. Su X, Lyu Y, Wang W, Zhang Y, Li D, Wei S, et al. Fascia Origin of Adipose Cells. Stem Cells. 2016;34:1407-19.
7. Nakagomi T, Kubo S, Nakano-Doi A, Sakuma R, Lu S, Narita A, et al. Brain vascular pericytes following ischemia have multipotential stem cell activity to differentiate into neural and vascular lineage cells. Stem Cells. 2015;33:1962-74.
8. Bianco P, Robey PG. Skeletal stem cells. Development. 2015;142:1023-7.
9. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. Circ Res. 2007;100:1249-60.
10. Khosla S. Minireview: the OPG/RANKL/RANK system. Endocrinology. 2001;142:5050-5.
11. Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, et al. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nat Biotechnol. 2004;22:560-7.
12. Esbrit P, Herrera S, Portal-Núñez S, Nogués X, Díez-Pérez A. Parathyroid Hormone-Related Protein Analogs as Osteoporosis. Calcif Tissue Int. 2016;98:359-69.
13. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9:669-76.
14. Deckers MM, Karperien M, van der Bent C, Yamashita T, Papapoulos SE, Löwik CW. Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. Endocrinology. 2000;141:1667-74.
15. Alonso V, Gortázar AR, Ardura JA, Andrade-Zapata I, Alvarez Arroyo MV, Esbrit P. Parathyroid homone-related protein (107-139) increases human osteoblastic celle survival by activation os vascular endothelial growth factor receptor-2. J Cell Physiol. 2008;217:717-27.
16. Liu Y, Olsen BR. Distinct VEGF functions during bone development and homeostasis. Arch Immunol Ther Exp (Warsz). 2014;62:363-8.
17. Liu Y, Berendsen AD, Jia S, Lotinun S, Baron R, Ferrara N, et al. Intracellular VEGF regulates the balance between osteoblast and adipocyte differentiation. J Clin Invest. 2012;122:3101-13.
18. Jilka RL, Weinstein RS, Bellido T, Roberson P, Parfitt AM, Manolagas SC. Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. J Clin Invest. 1999;104:439-46.
19. Jilka RL, O'Brien CA, Ali AA, Roberson PK, Weinstein RS, Manolagas SC. Intermittent PTH stimulates periosteal bone formation by actions on post-mitotic preosteoblasts. Bone. 2009;44:275-86.
20. Suda N, Gillespie MT, Traianedes K, Zhou H, Ho PW, Hards DK, et al. Expression of parathyroid hormone-related protein in cells of osteoblast lineage. J Cell Physiol. 1996;166:94-104.
21. Gimble J, Guilak F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. Cytotherapy. 2003,5:362-9.
22. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells; The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8:315-7.
23. Komori T. Regulation of osteoblast differentiation by transcription factors. J Cell Biochem. 2006;99(5):1233-9.
24. Song SJ, Jeon O, Yang HS, Han DK, Kim BS. Effects of culture conditions on osteogenic differentiation in human mesenchymal stem cells. J Microbiol Biotechnol. 2007;17:1113-9.

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Effects of the catalase antioxidant enzyme in vascular calcification and bone demineralization

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Summary

Objetives: Assess the role of the catalase antioxidant enzyme in the vascular calcification process associated with chronic renal failure (CRF) and its effect on bone mass.
Material and methods: Wild type C57/BL6J mice (WT) and transgenic mice (TG) were used, that overexpress the catalase enzyme, to which CRF was induced. Control WT and TG mice were used in simulated intervention. After 16 weeks, the mice were sacrificed, with serum samples taken for biochemical markers as well as residual pieces of kidney, aorta and tibias. An *in vitro* model of primary culture of smooth vascular muscle cells (SVMC) taken from the WT and TG aorta which underwent eight days of 3 mM phosphorus and 2 mM calcium calcifying medium.
Results: A significant increase in Runx2 gene expression, calcium renal deposit and bone structure deterioration at trabecular level was only detected in WT mice with CRF. This was not observed in TG mice with CRF.
Only in the case of WT mice SVMC, did added calcification medium raise calcium levels, proteic Runx2 expression and the reactive oxygen species of mitochondria with low catalase enzyme.
Conclusions: Calcifying catalase over-expression was observed in both *in vivo* and *in vitro*, with *in vivo* showing that this reduction was accompanied by an improvement in bone parameters under study.

Key words: *vascular calcification, bone, antioxidants, oxidative stress, catalase, µCT, chronic renal failure.*

Introduction

Cardiovascular disease currently represents the leading cause of death in the developed world. It is expected to continue rising in the coming decades due to our aging population. One factor that contributes to cardiovascular risk is oxidative stress[1]. Different stimuli are associated with the development of cardiovascular disease including macrophage activation, hyperglycemia, LDL oxidation and even angiotensin II which exert their harmful effects, at least partially, through the local synthesis of reactive oxygen species[2-5].

On the other hand, there is evidence of a positive relationship between oxidative stress and vascular calcifications[6]. Vascular smooth muscle cells (VSMC) subjected to oxidative stress increase the activity of alkaline phosphatase and calcium deposition, indicating their transdifferentiation toward cells capable of mineralizing (osteoblast-like/chondrocyte)[7]. In VSMC in primary culture of mouse aorta, the oxidative stress induced by hydrogen peroxide or glucose oxidase promotes calcification and overexpression of Runx2 (Cbfa1), transcription factor related bone osteogenic differentiation, mediated AKT[8]. Interestingly, these same stimuli have the opposite effect on osteoblast precursor cells, demonstrating the importance of hydrogen peroxide or oxygenated water (H_2O_2) in the process of differentiation of mesenchymal cells lineage[9].

The main antioxidant enzymes involved in the catalytic removal of hydrogen peroxide is catalase, glutathione peroxidases and thyroiodin peroxidase. Of these, the catalase is the most efficient in removing the hydrogen peroxide enzyme.

One of the factors affecting aging is the progressive accumulation of oxidative damage. This damage may be due to exposure to normal intracellular oxidative stress or, in pathological situations, an increase in this stress due to inflammation or other causes can actually cause accelerated aging. Therefore, our objective was to evaluate the role played by overexpression of catalase on the process of vascular calcification associated with moderate kidney disease and its effect on bone mass[10]. For this purpose, a transgenic mouse model overexpressing catalase enzyme, subjected to chronic renal failure was used.

Material and methods

Experimental model *in vivo*

Establishment of chronic renal failure (CRF)
C57/BL6J wild (WT) and C57/BL6J transgenic (TG) overexpressing the antioxidant enzyme catalase were used. To induce CRF, mice underwent 3 months old to a first intervention, which involved the lateral opening of the animal on the right side where the two poles of the kidney is cauterized. Isoflurane anesthesia was used (1-2%) by inhalation. A week after the first operation, the animal underwent a second intervention, which consisted of opening the left side and complete removal of the kidney. After 16 weeks of the last operation, the animals were sacrificed by exsanguination, anesthetized with CO_2. In slaughter animal serum he was obtained to analyze and general biochemical markers of bone metabolism: urea, calcium, phosphorus, Ca-P product, iPTH and FGF23. the remaining piece of kidney, aorta and tibias were also extracted.

The left tibia, which was preserved in ethanol 70°, was analyzed by computerized microtomography (μCT) with a SkyScan 1174, Bruker μCT (Kontich, Belgium) equipment. The 2D and 3D morphometric analysis was carried out using the CTAn software. The region of interest (ROI) was defined manually in each sample. For the trabecular region, 150 cuts were selected and threshold levels used in the gray scale between 78 and 250. The morphometric analyses were based on internal plug-ins CTAN in 2D and 3D. Morphometric parameters were measured trabecular bone volume (BV/TV,%), trabecular spacing (TbSp, μm), trabecular number (ToNb, mm-1) and trabecular porosity (TbPo, μm).

All studies were approved and authorized by the Committee on Animal Experimentation of the University of Oviedo.

In vitro experimental model

Primary cultures of aortic VSMCs from C57/BLJ6 WT and TG were used. To do this, the aortas of animals chopped and put explants to grow in culture dishes coated with fibronectin to promote adhesion.

Cells were cultured in DMEM medium supplemented with fetal bovine serum at 10%. When cells reached 60-70% confluency was replaced with DMEM-F12 supplemented medium with 0.1% bovine albumin (control medium) and calcifying medium supplemented medium consisting of control phosphorus and calcium at concentrations of 3 mM and 2 mM, respectively. Cells were incubated under these conditions for 8 days.

The basal activity of catalase was measured in VSMC from WT and TG mice using the commercial kit "catalase assay kit" (Cayman Chemical, 707002), following the protocol established by the manufacturer.

Levels of reactive oxygen species in VSMC cultured WT and TG mice with control medium and calcifying were measured with a fluorochrome specific mitochondria, dihydrorhodamine 123 (DHR 123).

Proteins of cell cultures were extracted with a buffer composition 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, sodium deoxycholate 0.5%, 1.0 mM EDTA and 0.1% SDS inhibitor proteases. the sample was sonicated in cold 10 minutes to prevent the breakdown of proteins and centrifuged at 10,000 rpm for 5 minutes at 4°C proteins of the supernatant was collected, quantified by the Bio-Rad DC method and stored at -80.

20 μg of protein were electrophoresed on acrylamide gels of 0.75 mm thickness under denaturing conditions (SDS-PAGE), for identification using known molecular weight markers (Molecular Weight Markers Rainbow™, GE Healthcare, United Kingdom). Proteins were transferred to a nitrocellu-

lose membrane (Hybond Amersham™ 0.45 µm PVDF, GE Healthcare, UK). The transfer was carried out in the cold for one hour at 100 v. After the same, the membrane was blocked for one hour with 5% milk in phosphate buffered saline (PBS). Subsequently, membranes were kept overnight with the primary antibody (monoclonal antibody of catalase in 1: 5000 dilution in BSA, Runx2 1: 500 in BSA against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) diluted 1: 5000 in BSA, all from Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA). The next day, the membrane was washed three times at intervals of 10 minutes each with wash solution composed of PBS and Tween-20 (Sigma-Aldrich), and subsequently incubated with antibody or goat against rabbit. The membrane was washed with the washing solution described above and the protein was detected by ECL Western Clarity™ Substrate (BIO-RAD, USA) commercial kit. The relative quantification of the intensity of the bands obtained in the Western blotting was performed with Image Lab™ Software and Molecular Imager scanner ChemiDoc™ XRS+ (both from BIO-RAD, USA).

Common techniques employed
qRT-PCR
The analysis by qRT-PCR was performed with the cDNA obtained from different experimental conditions using the High Capacity Reverse Transcription Kit (Applied Biosystems) and phenol RNA extraction. For normalization of results constitutive expression of genes (rRNA Runx2 and GAPDH) and $\Delta\Delta CT$ relative quantification method was used.

Study of vascular calcification
After addition of 0.6 N HCl for 24 hours to extract the cell or tissue calcium, the calcium content was quantified by ortho-cresolphthalein method complexone. Calcium levels were relativized to total protein content.

Statistical analysis
For the statistical analysis of the results the SPSS 17.0 software was used. Comparison of the treatment groups was performed using chi-square in the case of categorical variables and by Student's t test for numerical variables.

Results

a) *In vivo* studies:
Biochemical markers
The CRF in WT and TG mice showed increased levels of urea, Ca-P product, and iPTH FGF23. In the case of FGF23 increase it was much higher (10 times) in WT mice with CRF regarding their Sham control.

Moderate CRF effect on vascular calcification and bone level changes
Although the C57/BL6J mouse strain used for the calcification studies did not allow us to obtain vascular calcification in the aortas (Table 1), a significant increase in gene expression of Runx2, osteo-

blast differentiation marker, was observed only in the WT group with CRF (Figure 1). This increase was not observed in the TG animals with CRF. The effect of CRF on the calcification process was studied in other soft tissue such as the kidney, with an increase of calcium content in CRF mice with a higher increase in WT than in TG mice (Table 1).

Vascular level changes were also observed at bone level. In WT animals with CKD deterioration of bone structure it was about its Sham control group with a statistically significant decrease in trabecular bone volume (Figure 2A) and the number of trabeculae (Figure 2C), and increased trabecular separation (Figure 2B) and trabecular porosity (Figure 2D). The group TG mice with CRF did not differ with respect to their Sham control group at trabecular level.

b) *In vitro* studies:
Effect of overexpression of catalase in the hydrogen peroxide purification and Runx2 expression in VSMCs
The VSMCs from the TG mice had increased activity and protein expression of the catalase enzyme at baseline compared with those of WT mice (Figure 3 A and B).

When calcifying medium was added to VSMC WT mice there was increased calcium content. This effect was not observed in VSMC TG mice (Figure 4A). In parallel, the protein expression of Runx2 was studied in VSMC of WT and TG mice with calcifying medium for 8 days and an increase in Runx2 expression in VSMC of WT mice was observed which was not observed in TG (Figure 4B).

Study of the levels of markers of oxidative stress and protein levels of catalase
An increased level of fluorescence of the probe DHR123 was observed in the VSMC of WT mice cultured with calcifying medium, indicating an increase in mitochondrial oxygen reactive species. This effect was not observed in VSMC of TG mice, but there was a decrease of reactive oxygen species (Figure 5). Lower protein expression was also observed of the enzyme catalase by culturing the VSMC of WT mice for 8 days with calcifying medium relative to those from TG mice (Figure 6).

Discussion

In this work we have been able to confirm that overexpression of catalase antioxidant enzyme in a mouse model protects the process of vascular calcification and bone deterioration. In the VSMC of these same mice, a decrease was observed in the levels of reactive oxygen species, but also osteogenic proteins such as Runx2.

At the biochemical level, increased FGF23 was particularly noticeable in WT animals with CRF regarding the Sham group (10 times). This effect was less marked in the TG with CRF whose increase was twice that of the control group (Sham). Some authors have postulated the role of FGF23 as a calcification inducer[11,12]. It has even been associated with high levels of renal mortality in patients[13].

Table 1. Values of biochemical WT and TG mice with and without CRF

	WT Sham (n=7)	WT CRF (n=9)	TG Sham (n=10)	TG CRF (n=10)
Urea (mg/dL)	47±3	83±13*	41±4	94±19*
Ca-P product (mg²/dL²)	69±7	81±12*	60±14	74±13*
iPTH (pg/mL)	316 (0.834)	3,941 (3,649-4,499)*	338 (35-1,836)	3,411 (1,204-3,868)*
FGF23 (pg/mL)	111 (102-125)	1,103 (773-1,143)*	224 (204-268)	437 (153-988)
Calcium aorta (µg/mg)	92±7	95±6	82±11	93±5
Calcium kidney (µg/mg)	3.0 (1.9-4.0)	562.8 (200.2-636.3)*	0 (0-3.3)	20.7 (3.7-123.8)*

WT: wild mouse; TG: transgenic mouse; CRF: chronic renal failure. *$p < 0.05$ with respect to their respective Sham.

On the other hand, it inhibits FGF23 expression of CYP27B1 gene encoding the alpha 1-hydroxylase suppressing renal calcitriol synthesis from its precursor 25-hydroxyvitamin D3[14]. Furthermore, FGF23 activates CYP24 gene expression that encodes 24 hydroxylase, the enzyme that hydrolyzes and inactivates calcitriol[15]. This contributes to the decline in the synthesis of calcitriol, but also to degradation which leads to decreased levels of vitamin D, a factor that could induce vascular calcification as has been shown in epidemiological studies[16,17].

The calcium content in the aorta of TG mice with CRF was similar to WT mice with CRF. While this may seem paradoxical and contradictory to other observed results, Giachelli et al. have confirmed the absence of calcification in the aorta in the same strain of mice used for transgenic generation[18]. However, the protective effect of the overexpression of catalase preventing calcium accumulation was observed in other soft tissue such as the kidney.

Figure 1. Levels of gene expression of Runx2 (relative units) in aorta in different groups. WT: wild mouse; TG: transgenic mouse; CRF: chronic renal failure
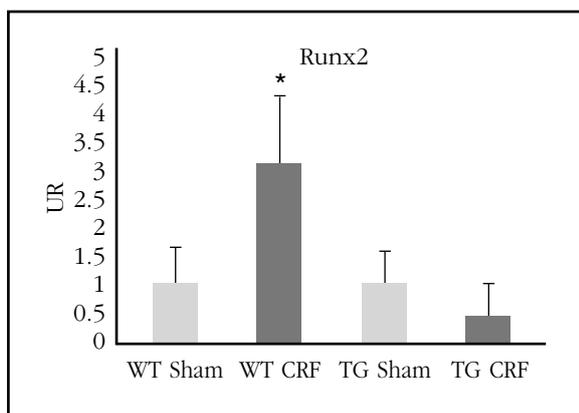
*$p < 0.05$ with respect to their respective Sham.

Moreover, high levels of FGF23 have been associated with suppression of osteoblast differentiation and mineralization of bone matrix *in vitro*[19]. This could perhaps explain the negative effect of CRF on bone deterioration in WT mice and to a much lesser extent in the TG mice. Recent studies by our group have shown bone loss in a rat model with CRF and high levels of phosphorus, PTH and FGF23. In the same study, gene silencing studies have confirmed that FGF23 only has a direct effect activating Dkk1[20], inhibitor of Wnt pathway which is involved in pathway inactivation. Thus, high levels of FGF23, as observed in our experimental model in WT mice with CRF, could have contributed to the decrease in bone mineral density through inactivation of the Wnt pathway.

The increase in reactive oxygen species contributes to increased osteogenic protein, being a stimulus for the start of the calcification process[7,8]. This effect has been observed in VSMC of WT mice subjected to calcifying stimuli. However, in VSMC of TG mice subjected to a calcifying stimulus, not only did the expression of reactive oxygen species not increase but it decreased, as with the protein expression of Runx2, osteogenic protein and early marker process calcification. In fact, the importance of hydrogen peroxide has noted as a second messenger involved in intracellular signaling[21] regulated by oxidative stress. A drop of hydrogen peroxide by catalase overexpression contributes to lower oxidative stress and decreased vascular calcification process through inhibition of Runx2.

In view of the results obtained, studies are needed to ascertain the mechanisms by which decreased oxidative stress confers an advantage at both vascular and bone level. The reduction of the inflammatory process, maintaining protein levels of renal Klotho, the main molecule involved in aging, or regulation of the Wnt pathway may help explain the different behavior, so further research into the field is required.

Figure 2. Changes to bone level; WT: wild mouse; TG: transgenic mouse; CRF: chronic renal failure. A) Trabecular bone volume (BV/TV) in different groups. *p<0.05 relative to its respective Sham. B) Trabecular separation (TbSp) in the different groups. *p<0.001 relative to its respective Sham. C) Number of trabeculae (TbN) in the different groups. *p<0.05 relative to its respective Sham. D) Porosity trabecular (PoTb) in the different groups. *p<0.05 relative to its respective Sham
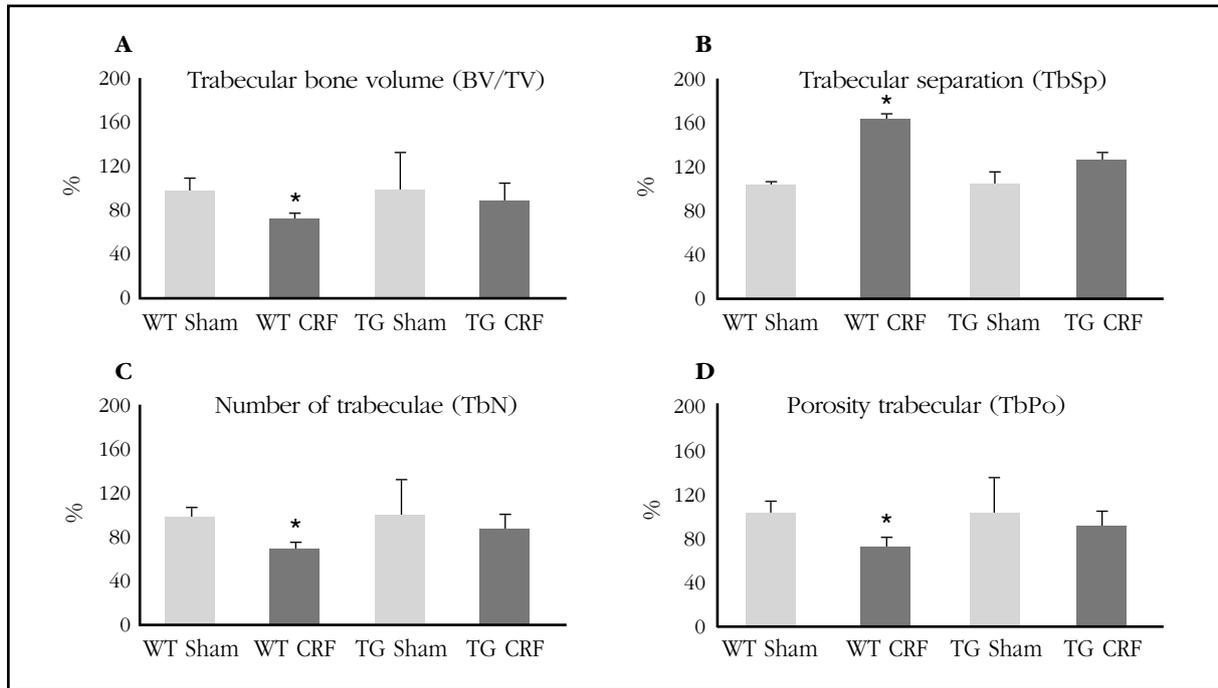
Figure 3. A) Basal Activity of catalase enzyme in WT and TG VSMC mice. B) Protein expression in relative units of catalase enzyme in VSMC WT and TG mice by Western blotting; WT: wild mouse; TG: transgenic mouse. *p<0.001 compared to WT group
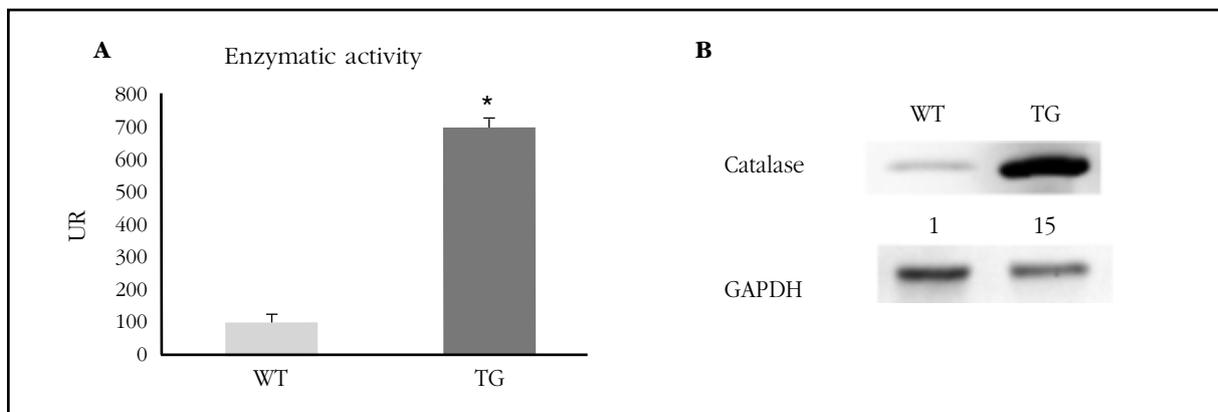
Figure 4. Effect of the addition of calcifying medium (Ca and P) in VSMC WT and TG mice on: A) The calcium content for 8 days. B) The protein expression of Runx2 for 8 days (the values of the densitometry vs GAPDH) are shown by Western Blot. WT: wild mouse; TG: transgenic mouse. *p<0.05 vs control
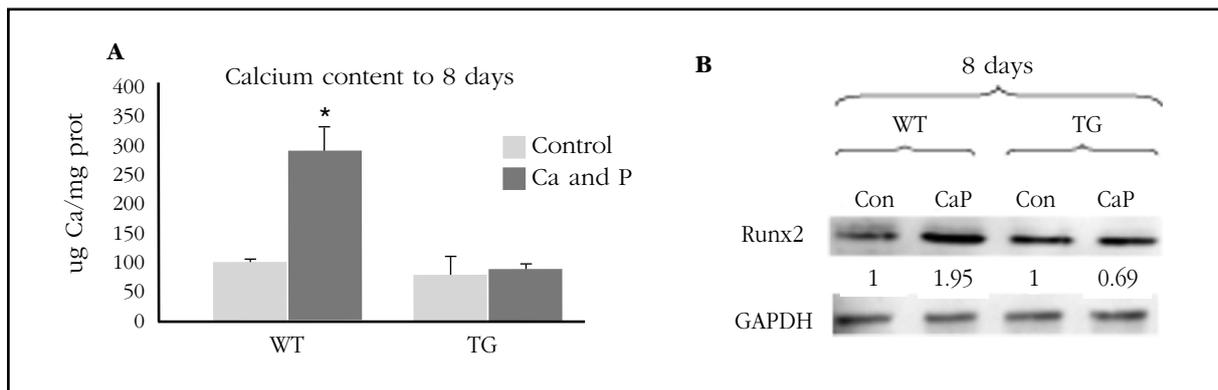
Figure 5. Effect of addition of calcifying medium (Ca and P) on the levels of reactive oxygen species in mitochondrial VSMC WT and TG mice. UR: relative units DHR123 fluorescence probe. WT: wild mouse; TG: transgenic mouse. *p<0.05 compared to control
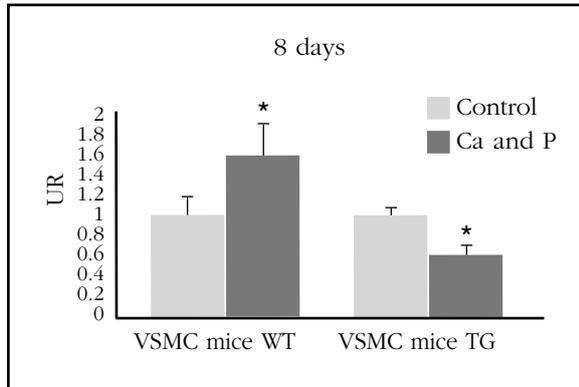
Figure 6. Effect of the addition of calcifying medium (Ca and P) in VSMC WT and TG mice on protein expression (relative units) of catalase at 8 days of culture by Western blotting. WT: wild mouse; TG: transgenic mouse
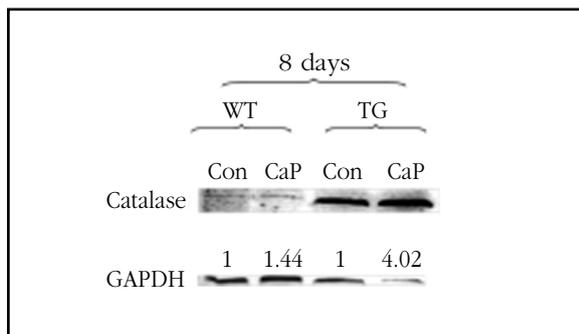
In summary, the overexpression of catalase enzyme reduced the calcification process both *in vivo* and *in vitro*, showing *in vivo* that this decline was accompanied by an improvement in bone parameters studied.

Declaration of interest: The authors declare no conflicts of interest.

Bibliography

1. Stocker R, Keaney JJ. Role of oxidative modifications in atherosclerosis. Physiol Rev. 2004;84:1381-478.
2. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. J Clin Invest. 1996;98:2572-9.
3. Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K, et al. Reactive oxygen species mediate the activation of Akt/Protein kinase B by angiotensin II in vascular smooth muscle cells. J Biol Chem. 1999;274:22699-704.
4. Uemura S, Matsushita H, Li W, Glassford AJ, Asagami T, Lee KH, et al. Diabetes mellitus enhances vascular matrix metalloproteinase activity: role of oxidative stress. Circ Res. 2001;88:1291-8.
5. Zmijewski JW, Moellering DR, Le Goffe C, Landar A, Ramachandran A, Darley-Usmar VM. Oxidized LDL induces mitochondrially associated reactive oxygen/nitrogen species formation in endothelial cells. Am Physiol Heart Circ Physiol. 2005;2:852-61.
6. Sutra T, Morena M, Bargnoux AS, Caporiccio B, Canaud B, Cristol JP. Superoxide production: a procalcifying cell signalling event in osteoblastic differentiation of vascular smooth muscle cells exposed to calcification media. Free Radic Res. 2008;42:789-97.
7. Mody N, Parhami F, Sarafian TA, Demer LL. Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. Free Radic Biol Med. 2001;31:509-19.
8. Byon CH, Javed A, Dai Q, Kappes JC, Clemens TL, Darley-Usmar VM, et al. Oxidative stress induces vascular calcification through modulation of the osteogenic transcription factor Runx2 by AKT signalling. J Biol Chem. 2008;283:15319-27.
9. Wang L, Zhang YG, Wang XM, Ma LF, Zhang YM. Narigin products human adipose-derived mesenchymal stem cells against hydrogen peroxide-induced inhibition of osteogenic differentiation. Chem Biol Interact. 2015;242:255-61.
10. Roman-Garcia P, Carrillo-Lopez N, Fernandez-Martin JL, Naves-Diaz M, Ruiz-Torres MP, Cannata-Andia JB. High phosphorus diet induces vascular calcification, a related decrease in bone mass and changes in the aortic gene expression. Bone. 2010;46:121-8.
11. Fang Y, Ginsberg C, Sugatani T, Monier-Faugere MC, Malluche H, Hruska KA. Early chronic kidney disease-mineral bone disorder stimulates vascular calcification. Kidney Int. 2014;85:142-50.
12. Jimbo R, Kawakami-Mori F, Mu S, Hirohama D, Majtan B, Shimizu Y, et al. Fibroblast growth factor 23 accelerates phosphate-induced vascular calcification in the absence of Klotho deficiency. Kidney Int. 2014;85:1103-11.
13. Gutiérrez OM, Mannstadt M, Isakova T, Rauh-Hain JA, Tamez H, Shah A, et al. Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. N Engl J Med. 2008;359:584-92.
14. Shimada T, Hasegawa H, Yamazaki Y, Muto T, Hino R, Takeuchi Y, et al. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. J Bone Miner Res. 2004;19:429-35.
15. Liu S, Tang W, Zhou J, Stubbs JR, Luo Q, Pi M, et al. Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. J Am Soc Nephrol. 2006;17:1305-15.
16. Dobnig H, Pilz S, Scharnagl H, Renner W, Seelhorst U, Wellnitz B, et al. Independent association of low serum 25-hydroxyvitamin d and 1, 25-dihydroxyvitamin d levels with all-cause and cardiovascular mortality. Arch Intern Med. 2008;168:1340–9.
17. Naves-Díaz M, Cabezas-Rodríguez I, Barrio-Vázquez S, Fernández E, Díaz-López JB, Cannata-Andía JB. Low calcidiol levels and risk of progression of aortic calcification. Osteoporos Int. 2012;23:1177-82.
18. Lau WL, Linnes M, Chu EY, Foster BL, Bartley BA,

Somerman MJ, et al. High phosphate feeding promotes mineral and bone abnormalities in mice with chronic kidney disease. Nephrol Dial Transplant. 2013; 28:62-9.

19. Wang H, Yoshiko Y, Yamamoto R, Minamizaki T, Kozai K, Tanne K, et al. Overexpression of fibroblast growth factor 23 suppresses osteoblast differentiation and matrix mineralization in vitro. J Bone Miner Res. 2008;23:939-48.

20. Carrillo-López N, Panizo S, Alonso-Montes C, Román-García P, Rodríguez I, Martínez-Salgado C, et al. Direct inhibition of osteoblastic Wnt pathway by fibroblast growth factor 23 contributes to bone loss in chronic kidney disease. Kidney Int. 2016;90:77-89.

21. Cozzolino M, Brenna I, Ciceri P, Volpi E, Cusi D, Brancaccio D. Vascular calcification in chronic kidney disease: a changing scenario. J Nephrol. 2011;24(Suppl 18):S3-S10.

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Influence of obesity on microarchitecture and biomechanical properties in patients with hip fracture

Summary

Introduction: Obesity and osteoporosis (OP) are two very prevalent diseases in our society today. The effect of obesity on bone quality is currently a subject under discussion.

Objective: To assess the effect of body weight on the microstructure and biomechanical properties of trabecular bone biopsies from the proximal end of the femur in patients with hip fracture fragility.

Material and Methods: Cross-sectional study of 16 patients with hip fracture. The 2 groups are divided according to their BMI: (A) normal weight individuals and (B) those with obesity. We collected biopsies of cancellous bone from the femoral head and assessed biochemical determinations (PTH, 25 (OH) vitamin D and IGF-1), bone remodeling markers (PINP, CTX), bone mass (BMD neck and total hip), bone microstructure and biomechanical study (µCt). Statistical analysis: Student's t test (SPSS 22.0) significance p<0.05.

Results: All patients had hip BMD in osteoporotic range. The obese group had higher levels of PTH and lower IGF-1, vitamin D and PINP. We found no differences in the parameters related to bone metabolism. The obese group showed better indices reaching microstructural significance: increased bone volume (BV/TV: 36.6±12.7 vs 19.4±11.4%, BS/TV: 5.5±1.1 vs 3.9±1.3%), higher trabecular number (Tb.N: 1.6±0.4 vs 1,01±0,4), greater trabecular width (Tb.Th: 0.22±0.003 vs 0.17±0.05) and lower trabecular separation (Tb.Sp: 0.51±0.12 vs 0.66±0.16). Biomechanical parameters confirm greater strength of trabecular bone in obese patients.

Conclusion: Obesity may be a protective factor of bone quality in the femoral region and has less effect on bone mineral density.

Key words: *osteoporosis, obesity, microstructure trabecular bone, biomechanics, bone mineral density, bone turnover markers.*

Introduction

Obesity and osteoporosis (OP) are diseases that have presented in epidemic form in recent decades. Both are of multifactorial etiology and chronic. They have a significant rate of morbimortality in developed countries[1,2]. The relationship between them has been studied extensively from different points of view, including epidemiological, clinical and basic research, and different links have been proposed, such as: 1) both are influenced by genetic and environmental factors, or the interaction between both; 2) aging is associated, with a high incidence of bone loss and adiposity in the bone marrow; 3) both bone remodeling and obesity are regulated by a complex of adipocytokines and hormones; 4) physical activity improves these two diseases; and 5) adipocytes and osteoblasts are derived from common progenitors[3].

Obesity is determined by an imbalance in which caloric intake exceeds consumption over a prolonged period[3,4] and constitutes a risk factor for hypertension, dyslipidemia, diabetes mellitus, cardiovascular disease and some forms of tumors[5].

Osteoporosis is a metabolic bone disease characterized by decreased bone strength due to a reduction in bone quantity and/or quality, which leads to an increased risk of spontaneous and traumatic fractures[6].

Traditionally, obesity has been considered a protective factor of bone loss and osteoporosis, because of the positive relationship between body weight, or body mass index (BMI), with bone mineral density (BMD), which is one of the main determinants of osteoporotic fracture risk[7]. This beneficial effect of body weight on BMD has been mainly related to increased bone formation due to mechanical loading, as well as the contribution of hormones derived from fatty tissue and its action on bone metabolism[6,8]. It has been shown that the incidence of hip fractures is decreased in subjects with a high BMI[9]. Likewise, low BMI (below 18 kg/m^2) reportedly presents a risk factor for osteoporotic fracture[10]. More recent evidence, however, indicates that while overweight (BMI between 25-29 kg/m^2) protects against OP, obesity (BMI >30) interferes with bone health[11]. Thus, fractures in children have been associated with alterations in body composition, such as an increase in adiposity and bone structure[12] and a risk of osteoporosis and non-vertebral fractures has been reported in subjects with the highest proportion of body fat, regardless of weight[11]. This has led researchers to suggest that the relationship between body mass index and obesity and the risk of frailty fracture and BMD is complex. A meta-analysis published in 2014 showed that more than 80% of osteoporosis fractures (including the hip) were found in women with a BMI <30 kg/m^2 [13] and in obese women, a higher prevalence of fractures of the proximal humerus and ankle[14,15].

Changes in parameters related to bone metabolism, such as insufficient levels of vitamin D and elevated PTH, have also been reported in obesity, along with markers of bone reshaping of formation and resorption[16], which points once again to a negative effect of fatty tissue on the bone. In addition, energy metabolism is closely linked to the osteoblastic response to insulin regulating homeostasis and bone remodeling. In stages of normaglycemia, insulin stimulates osteoblastogenesis and the production of RANKL inducing an increase in bone turnover. On the other hand, the release to the environment of decarboxylated osteocalcin regulates insulin production in an endocrine manner[17].

The effect of obesity on bone quality is currently under debate and very few studies have evaluated the microstructure and properties of bone in this condition. Thus, an inverse relationship between fat mass and the Trabecular Bone Score (TBS), assessed in the lumbar spine, was observed in premenopausal women with obesity, whereas there was no relationship with the structural analysis of the hip (HSA)[18]. A reduction of cortical bone has also been reported, due to a greater porosity, and yet an increase in volumetric trabecular BMD analyzed by peripheral computed tomography[19] and a negative correlation of cortical bone properties, resistance indexes (by microindentation), with BMI and total fat mass[20].

Our aim was to assess the effect of body weight on the microstructure and biomechanical properties of trabecular bone from femoral proximal extremity biopsies of obese patients versus subjects with normal weight who present fragility hip fracture.

Material and methods

1. Study design and subjects included

The present research is an experimental, analytical and transversal study of cases and controls. It was approved by the Virgen Macarena University Hospital Ethics Committee and informed, written consent was obtained from all participants. All included patients agreed to donate their bone samples for the study.

Patients have been included randomly. They entered our hospital's Unit of Clinical Management of Traumatology to be operated on for hip arthroplasty due to fracture or osteoporosis due to fragility or fall in height below that of the individual without acceleration mechanism, all were over 65 years of age. We included 16 patients divided into two groups according to BMI: 7 subjects formed group A with BMI <25, and 9 had group B with BMI >30.

We excluded patients taking medication with influence on bone metabolism (antiresorptive, osteoformers, corticoids and anticonvulsants) and/or vitamin D or calcium supplementation. The clinical history, densitometric, istomorphometric and biomechanical determinations were carried out.

2. Clinical data

A medical history was carried out that included data related to parentage; birthdate; anthropometric measures: weight (kg); height (cm) and body mass index (BMI) (weight in kg/height in m^2). History of osteoporosis and previous fractures were also included.

3. Biochemical determinations

Biochemical determinations, based on the serum samples, performed at the Biochemistry Service of the Virgen Macarena University Hospital (Seville).

Parameters included were: glucose, glycosylated hemoglobin (HbA1c), calcium, phosphorus, insulin-like growth factor 1 (IGF-I), total alkaline phosphatase (FAT), carboxyl terminal telopeptide of type I collagen (β-CrossLaps) and amino-terminal propeptide of collagen type I (P1NP) in auto-antibody ADVIA 2400 (Siemens). Vitamin D [25 (OH) D_3] and PTH were determined by chemiluminescent immunoassay (CLIA) on the CP ADVIA Centaur Immunoassay (Siemens).

4. Assessment of bone mass

We measured bone mineral density (BMD) of the lumbar spine (L2-L4) and hip (total hip and femoral neck) by Dual Absorptiometry X-Ray (DXA, Hologic-Discovery, Hologic Inc., Waltham, Massachusetts, USA). The CV *in vivo* was 1.40% (L2-L4 column), 2.9% (femoral neck) and 2.5% (total hip).

5. Bone histomorphometry and biomechanical study

Microstructural analysis of the biopsies was carried out using computerized microtomography (micro-CT), with SkyScan 1172, 100 kV, 1.3 MPixels. The entire sample was scanned to reconstruct the images and used for the quantitative and qualitative analysis of the trabecular bone microstructure. To analyze this microstructure, microtomography equipment software (CTAn 1.7.0.5) was used. The quantitative variables were: bone volume fraction (BV/TV), specific bone surface (BS/BV), bone surface density (BS/TV), trabecular thickness (Tb.Th), trabecular number), Trabecular pattern factor (Tb.Sp), trabecular pattern factor (Tb.Sp), trabecular pattern factor (Tb.Sp), anisotropy degree (DA), and structure-index structures.

The samples were subjected to a mechanical mono-axial compression test until rupture so as to evaluate the elastic-plastic mechanical properties of the biopsy (Microtest EM1/10/FR/m) at a constant loading speed and using a load cell of 1 kN or 10 kN, once the force-displacement curve was obtained Young's elastic modulus (E), the hardness (u), the maximum supported voltage (σ), the maximum force reached (F), the stiffness (S) and the energy required to fracture (U).

6. Statistical Analysis

The variables were analyzed for normal distribution by the Kolmogorov-Smirnov test. Student's t-test was performed to determine statistically significant differences between the two groups.

The SPSS version 22.0 package for Windows (IBM Corp., Armonk, New York, USA) was used for the statistical management of results. In all cases, the level significance was considered as $p < 0.05$. Data are expressed as mean ± SD.

Results

The anthropometric characteristics and BMD of the hip, femoral neck and spine are shown in Table 1. Both groups were similar in age, weight and lifestyle. Absolutely expressed BMD values and T-score of hip, femoral neck and spine were lower in the normal weight group, obtaining the greatest difference in hip T-score of -2.87±0,84 in subjects with normal weight and -1.67±1.07 in subjects with obesity although these differences were not statistically significant.

The FRAX® 10-year risk of major fracture and hip fracture was lower in obese patients than in patients with a BMI <25 kg/m², although it was not statistically significant.

The biochemical analysis of parameters related to bone metabolism is shown in Table 2. No differences were observed between the two study groups. It should be noted that vitamin D levels were found below 20 ng/mL in almost all patients studied independent of patients' BMI.

Microstructural indices show differences in the microarchitecture of spongy bone between both groups (Figure 1). The group of obese subjects presented higher BV/TV (p=0.015), BS/TV (p=0.015), Tb.Th (p=0.04) and Tb.N (p=0.007). In addition, they have less trabecular separation Tb.Sp (p=0.038) and lower values of Tb.Pf (p=0.015) and SMI (p=0.012). Indicating all this a better bone microstructure in the obese osteoporotic subjects compared to those who presented normal weight.

The biomechanical parameters studied (Figure 2) confirm a higher resistance of the trabecular bone in obese patients compared to subjects with normal weight. The obese group showed a greater rigidity, both in the stiffness due to the structural characteristics (p=0.029), and due to bone material properties: Young's modulus (p=0.01), maximum tension (p=0.036) and maximum force reached (p=0.034). In addition, the energy required to fracture the obese osteoporotic bone is twice that in subjects with normal weight, although this difference did not reach statistical significance.

Discussion

The effect of obesity on bone tissue is still unclear. Although it is known that obese women have reduced the volume of cortical bone and increased the volume of the trabecular bone[19], there are little data on the repercussion in the microstructure and/or biomechanics of the bone of obese people.

Our results indicate a positive effect of body weight on parameters of microarchitecture and biomechanics in trabecular bone. Although both study groups have presented hip fracture, with similar BMD values, bone quality characteristics are better in the obese group than in normal weight individuals.

The micro-CT study of the trabecular bone biopsies of the femoral head from obese fractured patients indicates that they present a greater amount of bone, in relation to the total body volume, greater bone density and greater number of trabeculae, and that these are wider. At the

same time, we also noted that the trabeculae have less separation between them. All these microstructural values correlate with the values obtained in the biomechanical studies in which we observe how the trabecular bone of the obese patients present a greater rigidity and a greater model of Young than the group of subjects with a BMI <25 kg/m^{2}.

We consider that this effect on the microarchitecture may be due to two fundamental facts: hormonal and/or mechanical factors. At the hormonal level, the increase in the aromatization of estrogens to androgens in adipose tissue leads to a decrease in sex hormones bound to globulin, a greater transformation of the adrenal hormones to peripheral estrones and hyperinsulinemia, which has a mitogenic effect on osteoblasts[21]. In addition, these patients, when carrying greater weight, have a greater mechanical activity on bone that may also stimulate osteogenesis[19,21-23].

The positive effect of body weight on bone tissue also leads us to question whether fatty tissue, muscle or both, to a greater or lesser extent, are responsible for these results. If it is the greater amount of fat or muscle tissue that is responsible for this skeletal beneficial effect, we cannot be sure, as we do not have data on hormone composition or serum levels of adipokines and myokines for these subjects.

Our results do not agree with those of other authors that indicate a worse bone microstructure of the femur in obese subjects[24]. However, these authors do not present results of bone biopsies but the microstructural values are evaluated by DXA. Recently, Shen et al., in a similar study, have not found such differences concluding that the adipose tissue can interfere in the values obtained since by DXA, the soft tissues that surround the bone, can give an erroneous reading in the measurement of the area Bone and therefore the amount of bone mineral content[25,26].

Obesity is associated with increased bone mass and a reduced risk of hip fractures. However, other fractures such as those of the ankle or humerus have a higher incidence in obese persons[27]. In our case, BMD was comparable in both study groups, the likelihood of fracture according to FRAX® was also similar between subjects with normal weight and obese, but we must consider that in all our hip fracture patients, there was no higher incidence of previous fractures in obese people than those of normal weight.

Our obese patients have slight increases in PTH levels and lower vitamin D and P1NP levels. In healthy post-menopausal women, PTH values correlate positively with BMI[19] and low levels of vitamin D are described. These lower levels of vitamin D have been attributed to a greater absorption of this hormone by the adipocytes[28] and to lower solar exposure, due to the more limited mobility of obese subjects[29]. On the other hand, it has been reported that obese patients tend to have less bone remodeling activity. It is unknown whether this is due to the effect of other diseases associated with obesity such as type 2 diabetes mellitus, among others, and/or by the effect of adipokines and myokines on remodeling[21,29].

Table 1. Anthropometric characteristics, FRAX® and BMD (mean ± standard deviation)

	Normal weight	Obese	p
Gender (♂/♀)	3/4	2/7	
Age (years)	78±9	79±7	
Height (cm)	157±9	153±8	
Weight (kg)	55.07±9.7	81.53±12.4	
BMI (kg/m²)	22.1±2.4	33.8±3.6	0.000
10 year risk of major fracture (FRAX®)	16.5±9.8	13.8±12.0	
10 year risk of hip fracture (FRAX®)	11.8±8.3	7.6±9.8	
BMD femur neck (gHA/cm²)	0.456±0.16	0.52±0.09	
BMD hip (gHA/cm²)	0.589±0.10	0.759±0.17	
T-score neck	-3.47±1.36	-3.02±0.64	
T-score hip	-2.87±0.84	-1.67±1.07	
BMD column (gHA/cm²)	0.81±0.05	0.86±0.85	
T-score column	-2.57±0.67	-2.17±0.64	

Table 2. Biochemical values (mean ± SD)

	Normal weight	**Obese**
Glucose (mg/dL)	98.33±33.5	129.5±32.5
HbA1c (%)	5.7±1.4	6.0±0.8
IGF-1 (ng/mL)	75.16±42.7	42.37±12.51
PTH (pg/mL)	46.5±32.3	76.4±46.7
β-CrossLaps (μg/mL)	0.8±0.5	0.7±0.3
P1NP (ng/mL)	89.4±57.5	54.1±36.5
Calcium (mg/dL)	9.6±0.5	9.1±0.1
Phosphorus (mg/dL)	3.6±1.1	2.9±0.4
Total alkaline phosphatase (U/L)	229.8±28.2	211.37±67.5
25-hydroxivitamin D (ng/mL)	12.9±9.1	9.3±4.6

Our study has several limitations: the main one is the sample size, which is relatively small, but the data obtained from microstructure and biomechanics, which were the objectives of our study, are quite forceful and statistically strong. We do not have serum levels of adipokines and hormones derived from the fatty tissue in order to relate them to the microstructural and biomechanical parameters.

In conclusion, we can say that measurements of trabecular bone biopsies from the femoral head indicate that obese patients have better biomechanical properties and better bone microarchitecture than patients with normal weight, showing a beneficial effect of body weight on bone quality.

Conflict of interests: The authors declare that there is no conflict of interests regarding this research paper.

Ethics: This study was approved by the Institutional Ethics Committee (HUV Macarena, Seville, Spain) and informed consent was obtained from each participant.

Bibliography

1. Mitchell NS, Catenacci VA, Wyatt HR, Hill JO. Obesity: overview of an epidemic. Psychiatr Clin North Am. 2011;34(4):717-32.

2. Hernlund E, Svedbom A, Ivergård M, Compston J, Cooper C, Stenmark J, et al. Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). Arch Osteoporos. 2013;8:136.

3. Migliaccio S, Greco EA, Fornari R, Donini LM, Lenzi A. Is obesity in women protective against osteoporosis? Diabetes Metab Syndr Obes. 2011;4:273-82.

4. Rössner S. Obesity: the disease of the twenty-first century. Int J Obes Relat Metab Disord. 2002;26(Suppl 4):S2-4.

5. Sowers JR. Obesity as a cardiovascular risk factor. Am J Med. 2003;115 Suppl:37S-41S.

6. Consensus development conference: prophylaxis and treatment of osteoporosis. Am J Med. 1991;90(1):107-10.

7. Ravn P, Cizza G, Bjarnason NH, Thompson D, Daley M, Wasnich RD, et al. Low body mass index is an important risk factor for low bone mass and increased bone loss in early postmenopausal women. Early Postmenopausal Intervention Cohort (EPIC) study group. J Bone Miner Res. 1999;14(9):1622-7.

8. Colaianni G, Brunetti G, Faienza MF, Colucci S, Grano M. Osteoporosis and obesity: Role of Wnt pathway in human and murine models. World J Orthop. 2014;5(3):242-6.

9. De Laet C, Kanis JA, Odén A, Johanson H, Johnell O, Delmas P, et al. Body mass index as a predictor of fracture risk: a meta-analysis. Osteoporos Int. 2005;16(11): 1330-8.

10. Mafi Golchin M, Heidari L, Ghaderian SM, Akhavan-Niaki H. Osteoporosis: A Silent Disease with Complex Genetic Contribution. J Genet Genomics. 2016;43(2):49-61.

11. Zhao L-J, Jiang H, Papasian CJ, Maulik D, Drees B, Hamilton J, et al. Correlation of obesity and osteoporosis: effect of fat mass on the determination of osteoporosis. J Bone Miner Res. 2008;23(1):17-29.

12. Migliaccio S, Falcone S, Spera G. Bone modeling and remodeling: from biology to clinical application. Aging Clin Exp Res. 2004;16 (Suppl 3):20-2.

13. Yu EW, Thomas BJ, Brown JK, Finkelstein JS. Simulated increases in body fat and errors in bone mineral density measurements by DXA and QCT. J Bone Miner Res. 2012;27(1):119-24.

14. Compston JE, Flahive J, Hosmer DW, Watts NB, Siris ES, Silverman S, et al. Relationship of weight, height, and

Figure 1. Comparison of structural parameters between patients with normal weight (A) and obese (B)
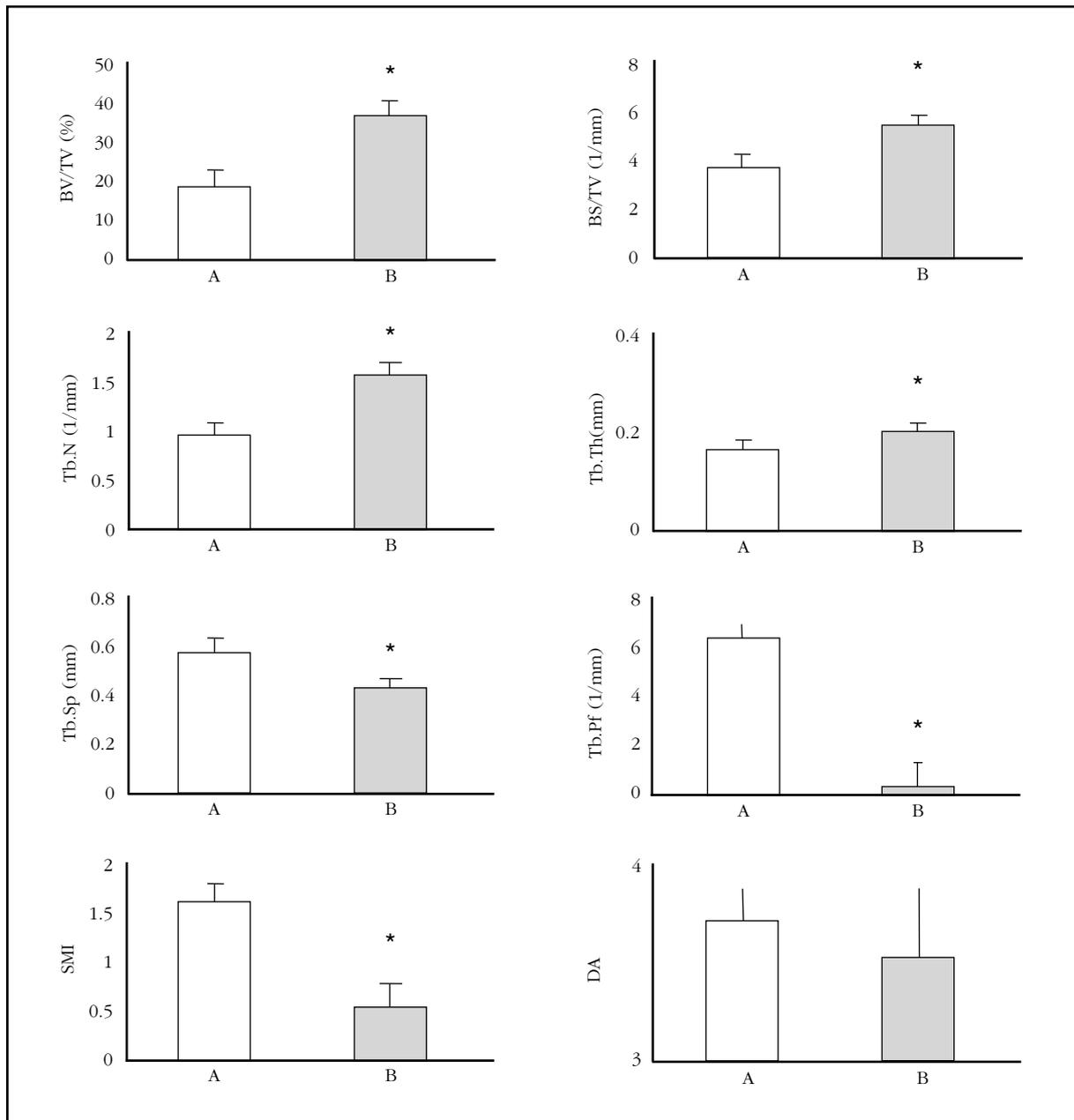
BV/TV: bone volume fraction; BS/BV: bone specific surface; BS/TV: bone surface density; Tb.Th: trabecular thickness; Tb.N: trabecular number; Tb.Sp: trabecular separation; DA: degree of anisotropy; SMI: Structural model index; Tb.Pf: trabecular connectivity.
Values are expressed as mean ± SD. *statistically significant values.

body mass index with fracture risk at different sites in postmenopausal women: the Global Longitudinal study of Osteoporosis in Women (GLOW). J Bone Miner Res. 2014;29(2):487-93.

15. Johansson H, Kanis JA, Odén A, McCloskey E, Chapurlat RD, Christiansen C, et al. A meta-analysis of the association of fracture risk and body mass index in women. J Bone Miner Res. 2014;29(1):223-33.

16. Schollenberger AE, Heinze JM, Meile T, Peter A, Königsrainer A, Bischoff SC. Markers of bone metabolism in obese individuals undergoing laparoscopic sleeve gastrectomy. Obes Surg. 2015;25(8):1439-45.

17. Lecka-Czernik B, Stechschulte LA. Bone and fat: a relationship of different shades. Arch Biochem Biophys. 2014;561:124-9.

18. Schorr M, Dichtel LE, Gerweck A V, Torriani M, Miller KK, Bredella MA. Body composition predictors of skeletal integrity in obesity. Skeletal Radiol. 2016;45(6): 813-9.

19. Sukumar D, Schlussel Y, Riedt CS, Gordon C, Stahl T, Shapses SA. Obesity alters cortical and trabecular bone density and geometry in women. Osteoporos Int. 2011;22(2):635-45.

20. Sundh D, Rudäng R, Zoulakis M, Nilsson AG, Darelid A, Lorentzon M. A. High amount of local adipose tissue is associated with high cortical porosity and low bone material strength in older women. J Bone Miner Res. 2016;31(4):749-57.

21. Palermo A, Tuccinardi D, Defeudis G, Watanabe M, D'Onofrio L, Lauria Pantano A, et al. BMI and BMD: The potential interplay between obesity and bone fragility. Int J Environ Res Public Health. 2016;13(6). pii: E544.

Figure 2. Comparison of biomechanical parameters between the two study groups: normal weight subjects (A) and obese (B)
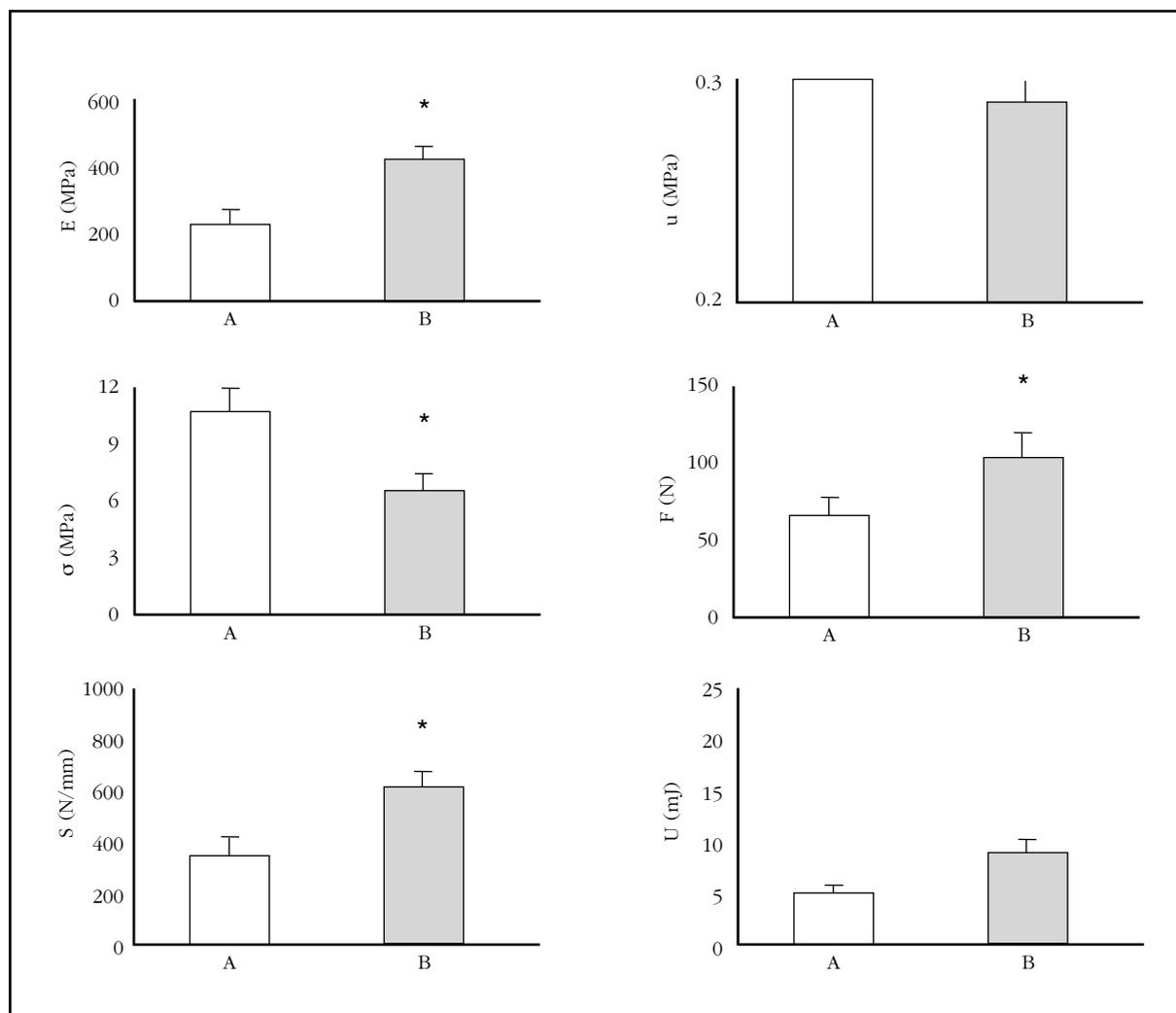
The parameters represented are: Young's elastic modulus (E); hardness (u); maximum supported voltage (σ); maximum force reached (F); stiffness (S); energy needed to fracture (U).

22. Evans AL, Paggiosi MA, Eastell R, Walsh JS. Bone density, microstructure and strength in obese and normal weight men and women in younger and older adulthood. J Bone Miner Res. 2015;30(5):920-8.
23. Lecka-Czernik B, Stechschulte LA, Czernik PJ, Dowling AR. High bone mass in adult mice with diet-induced obesity results from a combination of initial increase in bone mass followed by attenuation in bone formation; implications for high bone mass and decreased bone quality in obesity. Mol Cell Endocrinol. 2015;410:35-41.
24. Shen J, Leslie WD, Nielson CM, Majumdar SR, Morin SN, Orwoll ES. Associations of body mass index with incident fractures and hip structural parameters in a large canadian cohort. J Clin Endocrinol Metab. 2016;101(2):476-84.
25. Yu EW, Thomas BJ, Brown JK, Finkelstein JS. Simulated increases in body fat and errors in bone mineral density measurements by DXA and QCT. J Bone Miner Res. 2012;27(1):119-24.
26. Nelson L, Gulenchyn KY, Atthey M, Webber CE. Is a fixed value for the least significant change appropriate? J Clin Densitom. 2010;13(1):18-23.
27. Prieto-Alhambra D, Premaor MO, Fina Avilés F, Hermosilla E, Martinez-Laguna D,Carbonell-Abella C, et al. The association between fracture and obesity is site-dependent: a population-based study in postmenopausal women. J Bone Miner Res. 2012;27(2):294-300.
28. Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. Am J Clin Nutr. 2000;72(3):690-3.
29. Mpalaris V, Anagnostis P, Goulis DG, Iakovou I. Complex association between body weight and fracture risk in postmenopausal women. Obes Rev. 2015;16(3):225-33.

Roca-Ayats N[1], Cozar Morillo M[1], Gerousi M[1], Czwan E[2], Urreizti R[1], Martínez-Gil N[1], García-Giralt N[3], Mellibovsky L[3], Nogués X[3], Díez-Pérez A[3], Balcells S[1], Grinberg D[1]

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Identification of genetic variants associated with bone mineral density (BMD) in the *FLJ42280* gene

Summary

FLJ42280 is a possible gene for susceptibility to osteoporosis. Different studies of GWAs have identified 4 non-coding SNPs in this gene associated with bone mineral density (BMD) and fracture risk.

In order to ascertain the cause of the association between these SNPs and osteoporosis, we searched for genetic variants by resequencing the 28-kb gene, in a truncated selection of women with very low (n=50) or very high BMD (N=50) of the BARCOS cohort (Barcelona Cohort Osteoporosis, cohort of postmenopausal women in Barcelona). The variants found were filtered and their frequency analyzed in each group.

The overlap of the variants with functional elements of the ENCODE project was calculated. Finally, an eQTL analysis of the 4 SNPs-coding was performed on the expression levels of *FLJ42280* neighbor genes in lymphoblasts.

In all, 110 variants were selected. The differences in their frequencies between the two groups were below the statistical power of the experimental design. However, three variants overlapped with possible enhancers and one overlapped with an active enhancer in osteoblasts (rs4613908). A strong linkage disequilibrium was observed between the 4 non-coding SNPs and the SNP rs4613908, which belong to a block spanning the gene almost completely. None of the non-coding SNPs showed association with the expression levels of *FLJ42280* neighbor genes.

In conclusion, the SNP rs4613908 could be involved functionally in determining BMD. Tangible experiments will be required to confirm this.

Key words: *FLJ42280, bone mineral density, genetic variants, eQTLs, enhancers.*

Introduction

Osteoporosis is a complex disease characterized by low bone mass and deterioration of the bone tissue's microarchitecture which raises the risk of fracture. In the US, for example, there are 1.5 million new cases of fracture each year, thus incurring a huge economic burden for health care services. Osteoporosis is defined clinically by measuring bone mineral density (BMD), which remains the best means of predicting fracture[1,2]. Studies of heritability using twins or families have shown that 50-85% of the variation in bone mineral density is genetically determined[3]. Osteoporotic fractures also show independent heritability of bone mineral density[4].

Genome-wide association studies (GWAS) have greatly expanded our understanding of the genetic architecture of common and complex diseases[5]. This genomic approach is providing key information on disease mechanisms, with perspectives for designing more effective strategies for assessing disease risk and developing new therapeutic interventions[6]. However, genetic variants that are identified in GWAs are often found in non-coding regions of the genome whose possible function is less well known and in many cases these signals may be in linkage disequilibrium with non genotyped causal variants. The GWA meta-analysis for BMD and osteoporotic fracture of Estrada et al.[7] identified up to 56 genomic loci associated with BMD, 14 of which were also associated with osteoporotic fractures. One of the SNPs whose association with both phenotypes showed a more robust significance (rs4727338) was found in an intronic region of the *FLJ42280* gene, marking it as a locus of osteoporotic susceptibility (Figure 1). Other GWA studies showed that other intronic SNPs of the same gene (rs7781370, rs10429035 and rs4729260) were also associated with BMD[8,9]. *FLJ42280* is a gene which has not been studied extensively and its relation with bone biology is not known.

In this context, the aim of our work was to give meaning to this association by determining what the causal variant is. Is rs4727338 the causal SNP or is there another SNP in linkage disequilibrium with it that is the true functional SNP? To answer this question, we have explored the genetic variability of the genomic region where the *FLJ42280* gene is found and have addressed the functionality of these variants by different approaches. First, by resequencing the region in women with extremely high or extremely low BMD to look for variants with an unbalanced distribution between the two groups; Secondly, a bioinformatic study of the overlapping of the variants found with functional signals defined in the ENCODE project (The Encyclopedia of DNA Elements) and finally evaluate the possible role as eQTLs of some of the variants found.

Material and methods

Selection of study sample

The sample of this study consists of 100 women from the BARCOS[10] cohort. This cohort is composed of about 1,500 Spanish postmenopausal women monitored at the Hospital del Mar de Barcelona. Women diagnosed with osteomalacia, Paget's disease, some metabolic or endocrine disorder, or those undergoing hormone replacement therapy or drug treatment that could affect bone mass were excluded from the cohort. Women with early menopause (before age 40) were also excluded. The data collected for each sample were BMD, age, age of first menstruation, age of menopause, years since menopause, weight and height. Blood samples and informed, written consents were obtained from each patient according to the regulations of the Marital Health Park Clinical Research Ethics Committee. BMD (g/cm²) was measured at the femoral neck and lumbar spine. A dual energy X-ray densitometer was used to carry out the measurements.

Two groups of 50 samples with extreme BMD values were selected according to the Z-score value. Specifically, the groups consisted of the 50 samples with the highest Z-score (range: 2.98 to 0.73) and the 50 samples with the lowest Z-score (range: -2.41 to -4.26) of the BOATS cohort.

Preparation of genomic samples

Each woman's DNA was extracted from peripheral blood samples. The concentration and quality of the DNA samples (260/280 and 260/230 ratios) were measured by spectrophotometry on a NanoDrop ND-1000 (NanoDrop Products) instrument. To determine DNA integrity, 5 µl of each sample was analyzed by 1% agarose gel electrophoresis. Finally, the samples were normalized to a concentration of 100 ng/µl.

Long-Range PCR (LR-PCR)

A 28 kb genomic region (containing the *FLJ42280* gene [22 kb] along with 3.8 kb of flanking region at 5 'and 2 kb of flanking region at 3') was divided into 7 overlapping fragments (Figure 2). The sizes and coordinates of these 7 fragments and the primer pairs used to amplify them are shown in Table 1. The fragments, 2 to 5 kb, were amplified by LR-PCR. Each LR-PCR reaction included: 100 ng of genomic DNA, 5 µl of "Magnesium +" Ex Taq buffer (20 mM Mg++; Takara) x10, mixture of dNTPs (2.5 mM each), Ex Taq polymerase 5 U/µl) and primers (20 µM), in a final volume of 50 µl. The reactions were carried out in a GeneAmp® PCR System 2700 (Applied Biosystems) thermocycler. Each fragment required distinct elongation time and hybridization temperature conditions. The total number of amplicons was 700 (100 samples x 7 fragments). The quantity and quality of all amplicons were checked by 1% w/v agarose gel electrophoresis in TBE x1 buffer.

Purification and quantification of samples

To remove the residues from the PCR reagents, the PCR products were purified using 96-well filter plates with a suitable pore size (Pall Corporation). The vacuum (Vacuum Manifold, Merck Millipore) was applied and the DNA retained in the filter was resuspended in 35 µl of milliQ water. The PCR products

Figure 1. 7q21.3 genomic region with the BMD association signals of the SNPs tested by Estrada et al.[7]. The coordinate between the SNP genomic position rs4727338 (x-axis) and the -$\log_{10}$ p-value (y-axis) of its association with BMD (y-axis) is marked with a red line box. This SNP shows the greatest significance in this region. The colored points are the coordinates of the rest of the SNPs studied in the region. Each color indicates a different degree of linkage disequilibrium between each SNP and the SNP rs4727338. The *FLJ42280* gene is not shown because, at the time the GWAs meta-analysis was performed, this gene was not yet annotated in the genome. Its location between *SLC25A13* and *SHFM1* is indicated by a red oval. This figure is a modification of what is presented in Estrada et al.[7]

Technological Centers of the University of Barcelona, using the GS 454 Junior System (Roche). Briefly, the DNA was fragmented by nebulization, two labeled libraries were prepared with adapters (sequences of 10 nucleotides), one for each group, which were mixed in a single tube. The mixture was then amplified by emulsion PCR and the final library was loaded onto a picotiter plate for pyrosequencing. Four sequencing runs were carried out, corresponding to 140 Mb of final data (35 Mb/stroke). This volume of data provides a theoretical coverage of 40x for each initial sample.

Processing of sequencing data and variant selection
The readings obtained from the sequencing were preprocessed based on their quality and aligned against the reference genome (GRCh37) using the GS Mapper program (Roche). The readings were indexed and filtered using SAMtools. The variants present in the two groups were detected by GATK using standard filtering parameters[11]. The variants found were prioritized according to the following criteria: variants were selected with a coverage of at least 1,000 readings, present in 1% of readings and with a low bias strand. The number of readings of the variants that passed the filters was normalized by the coverage and the variants were classified between common (with a frequency greater than 5%) and rare or low frequency (with frequency less than 5%).

were then quantified using the Quant-iT PicoGreen dsDNA Reagent Kit (Life Technologies) following manufacturer's instructions. Briefly, a standard curve of concentrations was constructed by fluorescence emission measurements at 520 nm after the DNA was excited at 480 nm. The curve was then used to calculate the samples' DNA concentration.

Standardization of sample concentrations and pooling
The samples on the plates were normalized to a concentration of 5 ng/μl and then 5 μl of each sample of one plate (one tube per plate) was mixed in a single tube using the epMotion® 5075 Liquid Handling Workstation (Eppendorf). Thus, 14 tubes with 250 μl each were obtained, two for each PCR fragment (high BMD and low BMD). The 14 pools were pooled up to 5 times using the Genevac EZ-2 evaporator (Genevac SP Scientific) and each tube was quantified using Qubit® 2.0 Fluorometer (Life Technologies). Finally, the PCR fragments were mixed equimolarly in two tubes, one for high BMD and one for low BMD.

Massively parallel sequencing
The mass sequencing of the samples was carried out at the Genomics Service of the Scientific and

Functional and statistical analysis of variants
The frequencies of each variant were compared between the two groups using an exact Fisher's test, applying the Bonferroni correction for multiple comparisons. The functional analysis of variants consisted in looking at whether they were described in databases such as dbSNP and 1000 Genomes and, if so, searching for their MAF in the European and Iberian population. In addition, for the exonic variants it was observed what amino acid change they assumed and their severity predicted by SIFT, PolyPhen and Provean. For the intronic variants, we analyzed the region containing the variant: sites of hypersensitivity to DNAse, binding of transcription factors, DNA methylation, histone modifications and regulatory regions. All these data were obtained from databases and repositories such as Ensembl, UCSC Genome Browser, ENCODE, BioMart, MatInspector. HaploReg was also

used to search for regulatory annotations. Finally, all the variants found were analyzed with the Ensembl Variant Effect Predictor and UCSC and with the SNP function prediction from the US National Institute of Environmental Health Sciences.

Analysis of linkage disequilibrium

To calculate the linkage disequilibrium between all variants of the genomic region of *FLJ42280* we used the genotypes of the SNPs present in the region and the haplotypes of the individuals of HapMap phase 3. To calculate such an imbalance and generate a graph the software was used HaploView.

EQTL Analysis

The SNPs that gave significant differences in the GWAs and the SNP rs4613908 were assessed as possible eQTLs using two approaches: using the GTEx project portal and using the genotypes of those SNPs in HapMap individuals and the levels of cis gene expression in the same individuals. Specifically, the SNPs genotypes were obtained from 210 unrelated HapMap phase 1 and 2 individuals and the levels of expression of the *SHFM1*, *SLC25A13* and *DLX5* genes from a lymphoblastoid cell line from the same individuals obtained.

Results

Variants found and clues about its function

The genomic region of *FLJ42280* (28 kb) was massively resequenced in two DNA pools corresponding to the 50 women with the highest BMD and the 50 women with the lowest BMD of the BARCOS cohort (see details in Material and Methods above) at a high depth (about 3,600x per group). We compared the number and frequency of the variants found in each group. A total of 110 variants were identified, of which 18 were new and 59 were rare or low frequency variants (Table 2). It was observed that the number of low frequency variants between the two extreme groups was balanced. Likewise, it was observed that the frequency differences of all variants were below the statistical power of the design, although 9 showed a tendency.

For each variant, its overlap was analyzed with functional elements annotated in the genome by the ENCODE project. Four of the variants overlapped with possible transcription enhancing sequences (or enhancers) of osteoblasts and one of them [SNP rs4613908; MAF(CEU)=0.39] overlapped with an active enhancer in osteoblasts (Figure 3).

Linkage disequilibrium analysis

The linkage disequilibrium among all variants

Figure 2. In the genomic region of *FLJ42280* previous GWA studies have identified 4 SNPs showing association with BMD, details of which are shown in the table included in the figure. To better understand the variability of this locus, the region was subdivided into 7 overlapping fragments (yellow arrows) for resequencing in women with very high or very low BMD

	SNP	Alleles	MAF	Genome position GRCh37/hg19	Genome position GRCh38/hg38	Paper
SNP1	rs7781370	T/C	T=0.25	7: 96133531	7:96504219	Rivadeneira et al., 2009
SNP2	rs4727338	G/C	G=0.25	7: 96120675	7: 96491363	Estrada et al., 2012
SNP3	rs10429035	G/A	A=0.33	7: 96119481	7: 96490169	Zhang et al., 2014
SNP4	rs4729260	G/C	G=0.25	7: 96117918	7: 96488606	Rivadeneira et al., 2009
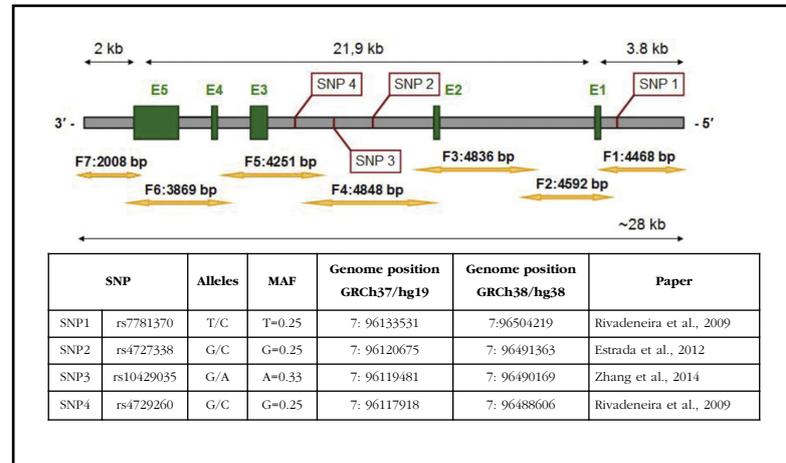
common in this region was also studied. We plotted linkage disequilibrium (LD) using HaploView and haplotype information from the HapMap project (Figure 4) and noted that there is a large LD block that includes almost the entire gene (with the exception of the 3 'UTR region) And that by the upstream part of the gene extends 5 kb beyond the resequenced region. It was also found that the SNPs rs4613908 and rs4727338 (GWAs meta-analysis of Estrada et al.[7]) present a large linkage disequilibrium between them.

eQTL Analysis

To complete the functional analysis, an eQTL analysis was carried out. Having the genotypes of the four SNPs associated with BMD, and the SNP rs4613908 of 210 individuals from the HapMap project and the levels of gene expression of a genomic array in lymphoblastoid lines from these same individuals, we determined whether the different alleles or genotypes Of the SNPs correlated with the levels of gene expression of the genes located in the genomic region of *FLJ42280*. None of the SNPs showed influence on the expression levels of the *SHFM1*, *SLC25A13* or *DLX5* genes (in the array there is no information on expression levels of *FLJ42280*). We also accessed the GTEx database to collect eQTL information for the same SNPs and the result was negative for all of them. Finally, we searched for regulatory annotations in HaploReg. This latter analysis confirmed that the sequence surrounding SNP rs4613908 is highly conserved among mammals and that in several cell types, including primary osteoblasts, contains chromatin markers typical of enhancer sequences (H3K4me1, H3K27ac). On the other hand, HaploReg highlighted alteration of regulatory motifs of this SNP and rs10429035, but showed no effect of these SNPs on gene expression.

Table 1. Amplicons used to sequence the *FLJ42280* region

Frag	Primers	Size (pb)	Genomic coordinates
1	**1F** TTGACCTGAATACTGCCGC **1R** GCCAAATGAATGTGGACAAG	4,468	7:96136619-96132152
2	**2F** CACTGCTGGGTCTTAGATTGG **2R** GCATGTGTGCATGATGTTGG	4,592	7:96132302-96127711
3	**3F** TGCAAGTTTCCCTCAATTCATC **3R** TCCCTCTCATCTGTGCAACAC	4,836	7:96127863-96123028
4	**4F** TTAGGTGAGTAGAAAGCAATGGC **4R** CTGGGTGGCTATAGACCTGAATAG	4,848	7:96123158-96118311
5	**5F** GCGGCACTGTGAGAGTACATC **5R** CCTGGTGGAAATGGGAACA	4,251	7:96118477-96114227
6	**6F** CTGACACTTTGGCAGCACC **6R** GGGATTGTTGAAGCTGACCC	3,869	7:96114348-96110480
7	**7F** CAACCATCACAACCCATAGAC **7R** CCTGAGCAAGTCTCGTAAGTG	2,008	7:96110702-96108695

Discussion

A comprehensive scan of a genomic region (28 kb) has been performed in 7q21.3 which contains several very strong association signals between 4 SNPs and bone mineral density[7-9]. It was wanted to know all the specific variants present in coding regions (exons of gene *FLJ42280*) and non-coding (introns, 3'UTR, 5'UTR and flanking gene) and to evaluate the functional potential of these variants to predict which of them could be responsible for the association with BMD. The variant rs4613908 has been shown to overlap with an active enhancer active in osteoblasts contained in a sequence with high evolutionary conservation. Said SNP (with its two allelic variants) could be affecting BMD by altering this gene enhancer. It remains to be determined which is the target gene of this enhancer.

To date, we have not found other studies which have addressed the functional basis of association with BMDs of SNPs located in non-coding regions of the *FLJ42280* gene. In fact, this gene has been recently annotated in the human genome, so that when the association of the SNPs of the region was detected, the gene was still missing on the map of 7q21.3 and the SNPs were left between the genes *SLC25A13* and *SHFM1* (Figure 1). Therefore, Estrada et al.[7] proposed that the functionality of the association could be related to *SLC25A13*. Currently, *FLJ42280* remains an annotated gene, with very few experimental data to confirm it. It is therefore very likely that the role of SNPs associated with BMD is related to other genes. In this sense, the *SHFM1* gene has been associated with some hereditary cases of cleft hand-foot malformation (Split hand and foot malformation 1; OMIM #183600) and the *DLX5* gene, below, is in fact the gene responsible for This disease, since there are patients with point mutations in *DLX5* that co-occur with the disease[12]. A number of enhancers have been described that affect the expression of *DLX5* in different tissues and stages of development and are distributed over several hundred kilo-bases. Studies in mice and zebrafish have characterized these enhancers and have been shown to function during development[13,14]. Some of them show tissue specificity and correlate with certain phenotypes present in patients with cleft-to-foot malformation carrying multiple chromosomal abnormalities (deletions or translocations) affecting the mentioned enhancers. By placing these *DLX5* enhancers on the map of the 7q21.3 region, we have seen with surprise that the SNP rs4613908, which we have just commented as a good functional candidate, is in one of these enhancers (eDLX#18), located at 500 kb of *DLX5*. The eDLX#18 enhancer has been described as active in the gill arches in embryonic stages[13].

There is evidence that *DLX5* is involved in the determination of BMD[15], suggesting that the eDLX#18 enhancer is also active as an enhancer for *DLX5* in adult osteoblasts and that our SNP of interest is an eQTL in osteoblasts. It will be crucial to test this hypothesis by analysis of *DLX5* expression in primary osteoblasts and genotyping of rs4613908 of the same.

Bibliography

1. Johnell O, Kanis JA, Oden A, Johansson H, De Laet C, Delmas P, et al. Predictive Value of BMD for Hip and Other Fractures. J Bone Miner Res. 2005;20:1185-94.

Table 2. Number and location of single nucleotide variants found in this study

	Raw	Filtered	Coding	Regulatory region*	Putative osteoblast enhancer	Active enhancer in osteoblast
Common variants	96	51	0	12	3	1
LFV	24.243	59	1	16	1	0
Total	24.339	110	1	28	4	1

*includes flanking regions, 5'UTR, 3'UTR and introns; LFV: low frequency variant (MAF <5%).

2. Kanis JA, Oden A, Johnell O, Johansson H, De Laet C, Brown J, et al. The use of clinical risk factors enhances the performance of BMD in the prediction of hip and osteoporotic fractures in men and women. Osteoporos Int. 2007; 18:1033-46.

3. Peacock M, Turner CH, Econs MJ, Foroud T. Genetics of Osteoporosis. Endocr Rev. 2002;23:303-26.

4. Deng HW, Chen WM, Recker S, Stegman MR, Li JL, Davies KM, et al. Genetic determination of Colles' fracture and differential bone mass in women with and without Colles' fracture. J Bone Miner Res. 2000;15:1243-52.

5. Hardy J, Singleton A. Genomewide Association Studies and Human Disease. N Engl J Med. 2009;360: 1759-68.

6. Manolio TA. Genomewide Association Studies and Assessment of the Risk of Disease. N Engl J Med. 2010;363:166-76.

7. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani E, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012;44,491-501.

8. Rivadeneira F, Styrkársdottir U, Estrada K, Halldórsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 2009;41:1199-206.

9. Zhang LL, Choi HJ, Estrada K, Leo PJ, Li J, Pei YF, et al. Multistage genome-wide association meta-analyses identified two new loci for bone mineral density. Hum Mol Genet. 2014;23:1923-33.

10. Bustamante M, Nogués X, Enjuanes A, Elosua R, García-Giralt N, Pérez-Edo L, et al. COL1A1, ESR1, VDR and TGFB1 polymorphisms and haplotypes in relation to BMD in Spanish postmenopausal women. Osteoporos Int. 2007;18:235-43.

11. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet. 2011;43:491-8.

12. Shamseldin HE, Faden MA, Alashram W, Alkuraya FS. Identification of a novel DLX5 mutation in a family with autosomal recessive split hand and foot malformation. J Med Genet. 2012;49:16-20.

13. Birnbaum RY, Everman DB, Murphy KK, Gurrieri F, Schwartz CE, Ahituv N. Functional characterization of tissue-specific enhancers in the DLX5/6 locus. Hum Mol Genet. 2012;21:4930-8.

14. Rasmussen MB, Kreiborg S, Jensen P, Bak M, Mang Y, Lodahl M, et al. Phenotypic subregions within the split-hand/foot malformation 1 locus. Hum Genet. 2016;135:345-57.

15. Ulsamer A, Ortuño MJ, Ruiz S, Susperregui AR, Osses N, Rosa JL, et al. BMP-2 Induces Osterix Expression through Up-regulation of Dlx5 and Its Phosphorylation by p38. J Biol Chem. 2008;283:3816-26.

Figure 3. A) Schematic of the genomic region of *FLJ42280* and location of some promising variants. Common variants are indicated in red and low frequency variants (LFV) or rare in gray. Blue squares represent enhancers of osteoblasts; The darker represents an active enhancer of the osteoblasts. B) Detail of active enhancer in osteoblasts. The sequence conservation profile in vertebrates, transcription factor binding sites and histone modifications are shown. These data were extracted from UCSC Genome Browser -GRCh37- and from ENCODE (data referred to osteoblasts)

Figure 4. Graph of linkage disequilibrium present in the genomic region of *FLJ42280*. A large block (24 kb) is seen that includes much of the gene except its 3'UTR region. The 4 SNPs that have been associated with BMD in the various GWAs are marked with green ovals. The SNP overlapping with the active enhancer in osteoblasts is marked with a purple oval. The blue horizontal line indicates the resequenced genomic region in this study
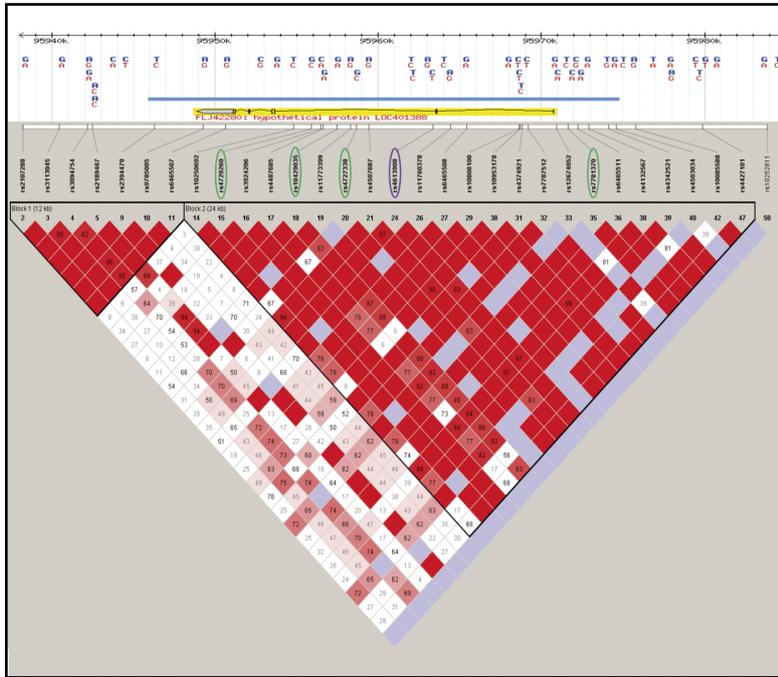
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Mesenteric panniculitis associated with the use of bisphosphonates: are these more proinflammatory than we know?

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Summary

Mesenteric panniculitis is characterized by chronic inflammation of the adipose tissue of the intestinal mesentery, and its etiology is unknown. It has been associated with malignancy, vasculitis, rheumatic diseases and the use of certain drugs. We present a case of panniculitis associated with bisphosphonate use, not previously described in the literature, thus suggesting its potential secondary proinflammatory effects.

Key words: *mesenteric panniculitis, bisphosphonates, proinflammatory.*

Introduction

Mesenteric panniculitis is a rare disorder characterized by chronic nonspecific inflammation of adipose tissue of the intestinal mesentery of unknown origin[1]. It may develop independently or in association with other alterations, which has been the subject of much discussion. It has been linked to various conditions, such as vasculitis, granulomatous diseases, rheumatic diseases, malignant diseases, pancreatitis[2], smoking and the use of certain drugs (beta blockers, methyldopa, sulfonamides, salicylates and oral contraceptives).

This entity has been found to present several stages of development. It is classified into different types depending on the radiological characteristics: type I (42%), diffuse thickening of the mesentery from root to the edges of the small intestine; Type II (32%), isolated nodular mass in mesenteric root; Type III (20%), the mesentery contains multiple nodules which vary in size[3]. Diagnostic anatomopathological criteria include: presence of large quantities of foamy macrophages in the initial phase of mesenteric lipodystrophy; Infiltrated plasma cells, giant foreign body cells, and foamy macrophages in mesenteric panniculitis. In the final stage, the deposition of collagen and the fibrosis in the tissues are noteworthy[4].

Although different factors have been recognized that may trigger the disease, its origin in certain cases remains uncertain. Once diagnosed, there is no specific treatment. Corticosteroids are recommended, although cases of spontaneous remission have been reported. Surgical resection is considered for cases in which there is intestinal obstruction due to inflammation and secondary scarring. Antibiotics, radiation therapy and cyclophosphamide have also been reported as possible responses.

Clinical Case Report

We present the case of a 67-year-old woman, without toxic habits, with no medical history of interest except cervico-artrosis and spondylolisthesis in rehabilitation and postmenopausal osteoporosis for 3 years following bisphosphonate treatment (BF). Mesenteric panniculitis was not justified by other causes.

Our patient, after 3 years of treatment with risedronate, began to present diffuse abdominal pain on a global basis, not related to meals and with episodes of frequent exacerbations over a period of about 2-3 months.

In one of the exacerbations, the patient was admitted and an abdominal computed tomography (CT) carried out, among other tests. In the CT, mesenteric panniculitis was revealed (Figure 1).

After reasonably ruling out other clinical etiologies of mesenteric panniculitis (Table 1)[5], the BFs that were taken and reevaluated at 6 months with a new abdominal control CT was taken, showing a complete resolution of the abdominal inflammatory lesions which presented in the previous CT scan (Figure 2).

Discussion

There are no similar cases described in the literature, nor have we found clinical trials of bisphosphonates, FIT, VERT, BONE, HORIZON[6-9], in cases of mesenteric panniculitis, although its use has been associated with other inflammatory processes. Cases of ocular side effects have been reported in this group of mostly inflammatory drugs such as uveitis, scleritis and conjunctivitis[10]. Conjunctivitis is the most frequent adverse ocular condition, although its actual incidence is very low. In general, it responds quickly to topical treatment, even if we maintain the drug, although it is more prudent to suspend it, at least temporarily. Another complication, less frequent but potentially more serious, is uveitis. Its incidence is very low, between 2 and 5 cases per 10,000 patients treated. In general, its location is anterior, although some isolated cases of posterior uveitis have been described. Its occurrence is quite variable, with a median number of days after the start of the 70-day drug (between 1 and 146) and its incidence is higher in patients treated with intravenous BF.

An increase in the susceptibility to uveitis in patients with associated diseases such as spondyloarthropathies, Behçet's syndrome, Wegener's granulomatosis or sarcoidosis and/or treatment with certain drugs has also been observed in which BP will act as a precipitating factor[11].

Bisphosphonates are potent inhibitors of osteoclasts by exerting a strong interaction with these cells, resulting in a marked decrease in bone resorption[12].

A number of adverse effects have been reported with bisphosphonate administration, which appear to correspond to "class effects." These effects may vary from bisphosphonates to others depending, among other factors, on the route of administration. Upper gastrointestinal tract such as esophagitis, gastric ulcers and even hemorrhages, musculoskeletal pain, influenza-like manifestations, atrial fibrillation, renal failure, hypocalcemia, maxillary osteonecrosis, atypical femoral fractures and ocular adverse effects such as conjunctivitis, uveitis, iritis, Episcleritis, scleritis or keratitis[13]. There has been no association with alterations of mesenteric panniculitis type adipose tissue in the literature.

Conclusions

Mesenteric panniculitis continues to be a nosological entity which is, in many cases, incidental and controversial. The retrospective analysis of many series has identified possible risk factors and a multitude of etiologic agents. Further studies and more described cases like ours are required to associate the use of BF more strongly with areas of inflammation of mesenteric adipose tissue. Although they have demonstrated their "proinflammatory" effect in other organs, especially at the ocular level, as we have developed previously.

Declaration of interest: The authors declare no conflicts of interest.

Figure 1. Abdominal CT scan showing area of panniculitis at the root of the mesentery

Figure 2. Abdominal CT scan 6 months later. There are no areas of mesenteric panniculitis
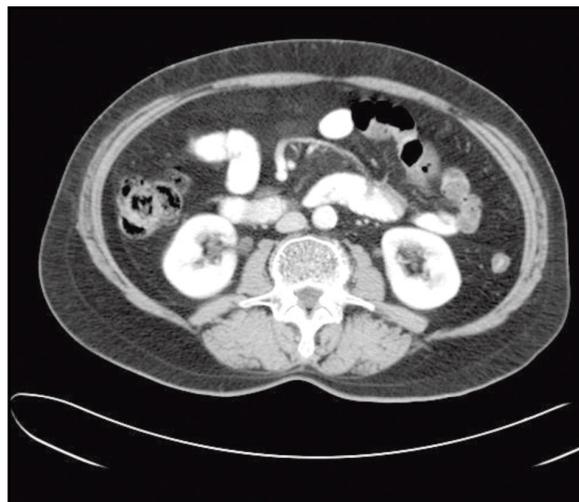
Table 1. Differential diagnosis of mesenteric panniculitis

Carcinoides tumors	Lymphomas
Reaction to adjacent cancer or chronic abscess	Lymphomas
Amyloidosis	Liposarcoma
Desmoid tumors	Peritoneal carcinomatosis
Infectious diseases (tuberculosis and histoplasmosis)	Peritoneal fibrosis
Peritoneal mesothelioma	Histoplasmosis
Lymphosarcomas	Retroperitoneal sarcoma
Chronic foreign body inflammation	Lymphangioma
Whipple's disease	

Bibliography

1. Ruiz Tovar J, Alonso Hernández N, Sanjuanbenito A, Martinez E. Paniculitis mesentérica: presentación de 10 casos. Rev Esp Enferm Dig. 2007;99:240-7.
2. Daskalogiannaki M, Voloudaki A, Prassopoulos P, Magkanas M, Stefanaki K, Apostolaki E, et al. Evaluation of mesenteric panniculitis: prevalence and associated diseases. Am J Roentgenol. 2000;174:427-31.
3. Colomer E, Blanes A, Carbonell C, Biot A, Villar H, Tomas A. Paniculitis Mesentérica con afectación retroperitoneal resuelta tras tratamiento con pulsos de ciclofosfamida endovenosa. An Med Intern. 2003; 20:31-3.
4. Delgado Plasencia L, Rodríguez Ballester L, López-Tomassetti Fernández EM, Hernández Morales A, Carillo Pallares A, Hernández Siverio N. Mesenteric panniculitis: experience in our center. Rev Esp Enferm Dig. 2007;99:291-7.
5. Viscido G, Napolitano D, Rivoira G, Parodi M, Barotto M, Picón H, et al. Paniculitis mesentérica asociada con absceso intraabdominal. Rev Chilena de Cirugía. 2008(3):452-6.
6. Harris S, Watts N, Genant H. Effects of risedronate treatment on vertebral and nonvertebral fractures in womenwith postmenopausal osteoporosis: a randomized controlled trial. JAMA. 1999;282:1344-52.
7. Black D, Thompson D, Bauer D, Ensrud K, Musliner MC, Hochberg MC, et al. Fracture risk reduction with alendronate in women with osteoporosis: the Fracture Intervention Trial. FIT Research Group. J Clin Endocrinol Metab. 2000;85:4118-24.
8. Chesnut III CH, Skag A, Christiansen C. Recker R, Stakkestad JA, Hioseth A, et al. Effects of oral ibandronate administered daily or intermittently on fracture risk in postmenopausal osteoporosis. J Bone Miner Res. 2004;19:1241-9.
9. Black DM, Delmas PD, Eastell R, Reid IR, Boonen S, Cauley JA, et al. Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis. N Engl J Med. 2007;356:1809-22.
10. Cano Parra J, Díaz-Llopis M. Uveitis Inducida por fármacos. Arch Soc Esp Oftalmol. 2005;80:1-12.
11. Arboleya L, Alperi M, Alonso A. Efectos adversos de bifosfonatos. Reumatol Clin. 2011;07:189-97.
12. Jaimes M, Chaves Netto HD, Olate S, Chaves MMGA, Barbosa AJR. Bifosfonatos y osteonecrosis de los maxilares. Consideraciones sobre su tratamiento. Int J Morphol. 2008;26(3):681-8.
13. González-Macías J, del Pino-Montes J, Olmos JM, Nogués X. Guías de práctica clínica en la osteoporosis posmenopáusica, glucocorticoidea y del varón. Sociedad Española de Investigación Ósea y del Metabolismo Mineral. (3ª versión actualizada 2014). Rev Clin Esp. 2015;215:515-26.

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Osteoporosis in rheumatic diseases and glucocorticoid induced

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Summary

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density, changes in bone microarchitecture and increased risk of fracture. It has been shown that depends on physiological processes and secondary to other pathologies and associated with the use of glucocorticoids, the latest being the most common cause of osteoporosis associated to drugs, this may be represent a great magnitude public health issue. This review is presented in order to emphasize the clinical importance of osteoporosis in rheumatic diseases and glucocorticoid-induced osteoporosis.

Key words: *osteoporosis, rheumatic diseases, glucocorticoids.*

Introduction

Osteoporosis is a systemic skeletal disease characterized by decreased bone mineral density with alterations in bone microarchitecture and increased risk of fracture, involving genetic and environmental factors. It is classified as primary when it depends on physiological processes, as in the case of menopause and normal aging, and secondary when conditioned by other pathologies or in relation to medications, as in osteoporosis secondary to rheumatic diseases and glucocorticoid-induced osteoporosis (GIO).

The current trend of widespread corticoid use has led to GIO being the most common cause of drug-induced osteoporosis, constituting a worldwide problem. Approximately 0.5% of the general population and 1.7% of women over 55 years of age receive oral glucocorticoids and, despite the existence of diagnostic methods and adequate measures to prevent GIO, less than 14% receive some type of treatment to avoid loss of bone mass due to glucocorticoids[1-3].

In patients with rheumatic diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), ankylosing spondylitis (AS), polymyalgia rheumatica (PMR), and vasculitis, among others, osteoporosis is an associated comorbidity. This may be due to the use of glucocorticoids over long periods of time as part of the accepted therapy of these diseases, or it may also be associated with the inflammatory activity of the condition and its impact on the bone. In any case, these patients are more likely to suffer fractures due to fragility, which leads to a decrease in the quality of life[3,4].

The clinical relevance of osteoporosis in rheumatic diseases is underestimated. Studies have shown that patients with RA receiving oral glucocorticoids who underwent a control bone densitometry had osteopenia/osteoporosis in 23% of the cases, of which 42% were prescribed at least one medication to reduce bone loss[3].

This indicates an insufficient appreciation of the clinical problem and a lack of consensus on osteoporosis detection and treatment in rheumatic diseases.

Effects of inflammation on bone turnover

The determinants of inflammation on the bone are proinflammatory cytokines. Thus, the alpha (TNF-α) tumor necrosis factor, interleukins (IL) 1 and 6 (IL-1 and IL-6) favor bone resorption by directly or indirectly promoting osteoclastogenesis. IL-17 enhances activator receptor ligand expression for nuclear factor kappa B (RANK-L), which in turn is a key part of the bone resorption process (Figure 1).

TNF acts on proinflammatory signaling pathways within the joints, influencing bone turnover, at the same time as it seems to be involved in developing bone erosions and the progression of osteoporosis. In addition, TNF has been shown to promote pre-osteoclastic cell formation expressing the activator receptor for nuclear factor kappa B (RANK), promoting overexpression of RANK-L osteo-protegerin (OPG) and inhibiting the maturation and function of the osteoblasts, because it increases the expression of the synovial fibroblasts[5].

Figure 1. Regulation and formation of osteoclasts
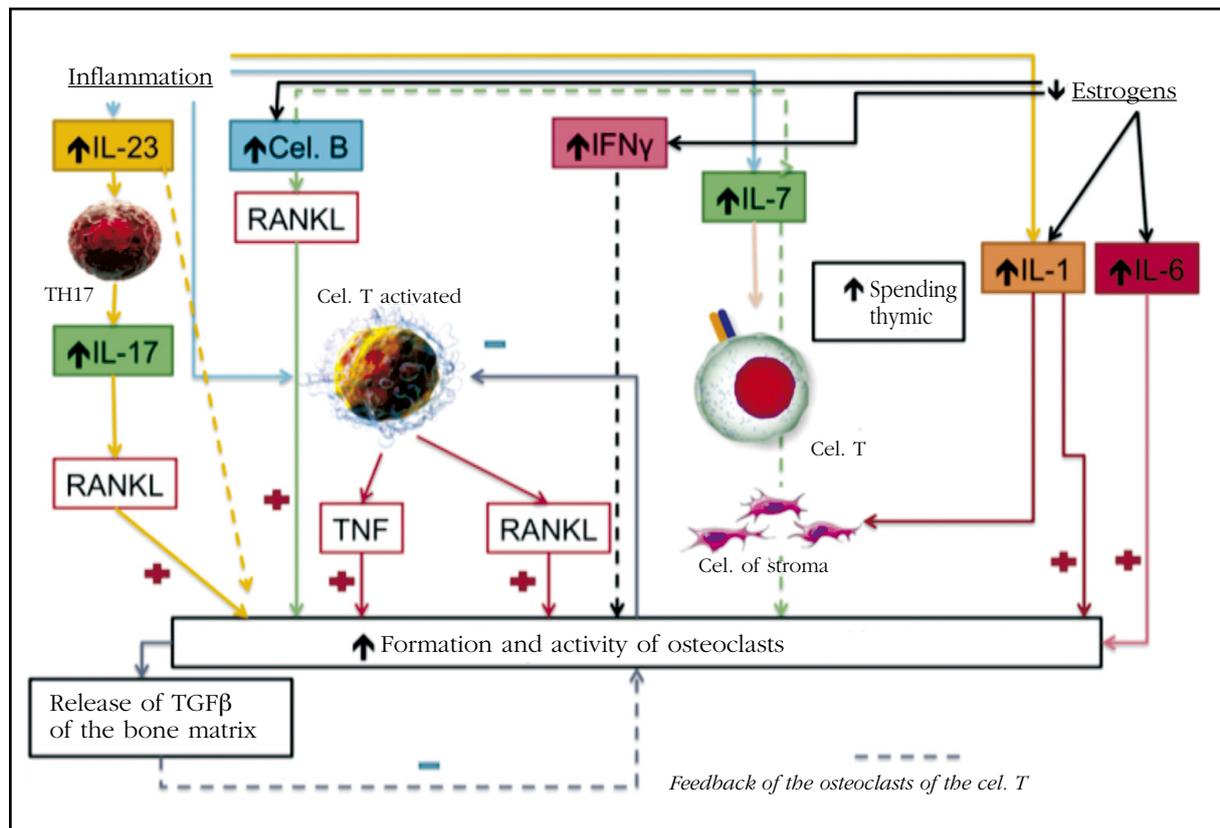
The relationship between proinflammatory cytokines and resorption is well established. However, there is a lack of evidence on the relationship between cytokines and bone formation. IL-4, IL-12, IL-8 and interferon are known to promote bone formation due to increased OPG/RANKL ratio, thus inhibiting osteoclastogenesis[6].

On the other hand, the osteoblast produces cytokines and in its immature state promotes the formation of RANK-L and in its mature state the production of OPG, showing that the osteoblast maturation process is essential to balance the relationship between the inflammatory process and bone mass[6].

Rifas and Weitzmann discovered a cytokine and called it a secreted osteoclastogenic factor of activated T cells (SOFAT)and demonstrated that it induces the osteoblastic production of IL-6 and the formation of osteoclasts in the absence of osteoblasts or RANKL, and that it is insensitive to OPG effects. This demonstrates that SOFAT is a potent inducer of IL-6 production, playing a key role in the local inflammatory response, and may indirectly exacerbate bone destruction in rheumatoid arthritis through multiple IL-6 mediated processes[7].

Acute and chronic inflammatory processes in rheumatic diseases cause bone damage because inflammation increases the number and activity of osteoclasts and, at the same time, the activation of T cells. During infectious processes increases RANKL production, which, in turn, stimulates osteoclastogenesis. It has been demonstrated that the application of OPG can reverse this effect in diseases such as RA and periodontitis[8].

Osteoporosis in specific rheumatic diseases

It is known that subjects with a history of chronic autoimmune diseases have a higher risk of developing osteoporosis due to different causes, such as specific alterations that affect bone metabolism, induce loss and sometimes inhibit formation, or to meet factors such as: sex (predominance in women), low physical activity, underlying disease, smoking, low body mass index (BMI), vitamin D deficiency, etc. (Table 1).

The Multidisciplinary Forum on Management of Patients with High Risk of Osteoporotic Fracture (HRF) has suggested classifying risk factors as follows[9]:

- **Keys:** age over 70 years, history of previous frail fracture (vertebral or hip), glucocorticoid intake (7.5 mg/day for 3 months or more) and BMD (T-score ≤3).

- **Important:** maternal history of hip fracture, BMI ≤20, frequent falls in the elderly, low measurements in activity and physical function.

- **Moderate:** levels of 25(OH)-vitamin D ≤30 ng/dl, some lifestyle-related harmful factors (smoking, excessive alcohol intake, sedentary lifestyle or excessive coffee consumption).

Rheumatoid arthritis (RA)

RA is an autoimmune inflammatory disease of unknown origin that is more common in young women, between the third and fifth decades of life [4]. It is a deforming and disabling disease that directly affects the joint causing bone erosions, which end up injuring the joint and resulting in its deformity.

Osteoporosis is a common complication of RA and is classified as follows[10]:

- **Yuxta-articular:** early and secondary to local factors.

- **Generalized:** late and multi-causal in origin (rest, drugs, disease activity, degree of functional alteration and deterioration of muscle mass).

The frequency of osteoporosis in RA is variable. Sinigaglia et al reported an incidence of osteoporosis of 10-56% in patients with RA[3]. Forsblad added that this range will depend on the population studied, and stressed the importance of joint damage in RA and generalized osteoporosis[11].

Patients with RA have a higher risk of fracture compared to the general population. A retrospective study of 30,262 patients showed a higher risk of hip fracture (RR=2.0) and spine (RR=2.4) vs the control group, and the risk increased due to prolonged use of corticosteroids (RR=3.4)[12].

The high risk of fracture in RA patients has also been associated with disease-specific factors[13] (Table 2).

Corticosteroid use is an independent predictor of the decrease in bone mass at the lumbar and femoral levels. A low dose steroid regimen (5 mg prednisone) is associated with a 50% increase in the risk of osteoporosis[14]. This demonstrates that treatment with oral corticosteroids of more than 5 mg of prednisone is able to reduce bone mass with a rapid increase in the risk of fracture during the treatment period[15,16]. Taking into account this risk factor, an American College of Rheumatology (ACR) committee recommends screening for osteoporosis using BMD and initiating treatment in patients with T-score ≤1.0, preferably with bisphosphonates and calcium supplements and Vitamin D. These patients should be monitored annually and treatment should continue while receiving glucocorticoids[17].

Longitudinal and cross-sectional studies have shown that disease modifying anti-rheumatic drugs (DMARDs), such as methotrexate, do not have adverse effects on bone mass[18]. Anti-TNF therapy (infliximab) has shown positive effects on bone mass[19]. It is believed that the bone protective effect is not only due to the decreased disease activity, but also to the role played by the cytokine TNF in osteoclastogenesis[20,21]. Regarding tocilizumab (inhibitor of IL-6), its role is fundamental, since IL-6 intervenes in the inflammatory and osteoclastogenic process[13].

The CAMERA II study analyzed patients with early RA who were on methotrexate and 10 mg prednisone and were administered 500 mg of calcium, 400 IU of vitamin D and alendronate/risedronate over two years. BMD showed a 2.6% increase in lumbar spine bone mass during the first year of prophylactic treatment regardless of glucocorticoid use, demonstrating that the addition of 10 mg of prednisone daily in a methotrexate-based treatment does not lead to bone loss in patients with early RA[22].

The quality of life of these patients is obviously affected. A study by Riggs and Melton showed that a hip fracture has a mortality rate of 10-20% in the sub-

sequent 6 months, 50% of these patients will not be able to walk without external objects such as walking sticks. Also 25% will need home help for long periods[23].

Systemic Lupus Erythematosus (SLE)

SLE is an autoimmune disease of unknown origin that occurs more frequently in young women[4]. Osteoporosis is also an associated disease, and probably caused by treatments that have a negative effect on the bone or by ovarian dysfunction induced by some immunosuppressants[24].

The loss of bone mass in SLE could be the result of several mechanisms, those that depend on the disease itself and those that are treatment related (Table 3).

The mechanism responsible for the loss of bone mass in patients with SLE receiving corticosteroids is the marked increase in bone resorption and the maturation deficit of the osteoblast, as well as bone mineralization. BMD begins to fall from the third month of glucocorticoid use and progresses rapidly to six months. From this period, the curve gradually declines[25].

A study by Houssiau et al in premenopausal women with a definitive diagnosis of SLE, showed that patients who had not received corticosteroids had lower BMD compared to healthy controls, indicating that the disease alone is a bone loss factor[26].

A systematic review by Wang et al showed that SLE patients have lower levels of BMD than the general population, and that not only occurs in a specific place, but there is loss of bone mass in all the places studied (femoral neck, lumbar spine and hip). In addition, an increased risk of fracture was demonstrated (RR=1.97)[27].

Jacobs et al, in a prospective 6-year follow-up study in patients with SLE, found an association between loss of BMD in the lumbar spine and the use of corticosteroids at high doses. In addition, a loss of bone mass was demonstrated with the use of immune-suppressants and base antimalarials[28].

Regarding the association between lower serum vitamin D levels in these patients and the loss of bone mass, the deficiency is believed to be due to reduced sun exposure by the photosensitivity of these patients, in addition to the use of corticosteroids and renal insufficiency[29]. The mechanism by which these patients have a lower calcium absorption results in a decrease in the conversion of 25(OH)-vitamin D to $1,25(OH)_2$-vitamin D (calcitriol). This association has been demonstrated in cross-sectional studies[29-31], hence the importance of determining levels of 25(OH)-vitamin D in patients with SLE.

Table 1. Profile of high risk of fracture (HRF). Group of Rheumatology

Factors associated with HRF
✓ Age >70 years ✓ Fracture due to previous fragility (symptomatic or asymptomatic) ✓ Low BMD (T-score <3) ✓ Maternal history of hip fracture ✓ Administered corticoids ≤7,5 mg/day over more than 3 months ✓ Low weight (BMI <19 kg/m²)
Special and common situations in rheumatologic patients
✓ Chronic inflammatory disease with persistent activity ✓ Rheumatic polymyalgia and/or giant cell arteritis ✓ Transplants (distinguishing between pre-transplant and post-transplant) ✓ Frequent use of treatments that induce osteoporosis
HRF in patients with rheumatologic diseases
Osteoporosis induced by glucocorticoids ✓ Daily dose of corticosteroids greater than 15 mg ✓ Treatment time greater than one year **Rheumatoid arthritis:** ✓ Postmenopausal women (especially those over 65 years of age) ✓ DMO similar to the risk of postmenopausal osteoporosis ✓ Treated with corticosteroids at doses higher than 15 mg/day ✓ High disability rate ✓ Extended disease ✓ Low physical activity **Ankylosing spondylitis:** ✓ Patients with a disease of more than 10 years of evolution ✓ Male >30 years old, treated with corticosteroids ✓ Accused loss of BMD in the first 5 years, with an extended disease ✓ Back pain episodes in the last 6 months ✓ Associated inflammatory bowel disease **Systemic lupus erythematosus:** ✓ Postmenopausal woman, with a long-term disease ✓ Start after age 30 ✓ Low sun exposure ✓ Uses sunscreens ✓ Low hip BMD **Systemic sclerosis:** ✓ Age >50 years ✓ Female ✓ Early menopause ✓ Body mass index <25 ✓ Use of systemic corticosteroids **Rheumatic polymyalgia/giant cell arteritis:** ✓ Age >60 years ✓ Functional limitation (low physical activity) ✓ Use of systemic corticosteroids (high cumulative dose) ✓ Decrease in strength ✓ Reduced BMD

Table 2. Profile of high risk of fracture (ARF). Rheumatoid arthritis

Very relevant
✓ Age i>70 years
✓ Low BMD (T-score <3)
✓ The previous existence of at least 2 vertebral fractures or hip fracture
Relevant
✓ More than 2-3 falls per year
✓ Use of oral corticosteroids at a dose of 7.5 mg/day for at least 3 months
✓ BMI <19 kg/m
✓ Family history of hip fracture
✓ Tobacco consumption >10 cigarettes/day

Table 3. Possible mechanisms of loss of bone mass in SLE

Dependent on disease	Dependent on treatment
✓ Reduced mobility	✓ Long-term corticosteroids
✓ Renal insufficiency	✓ Immunosuppressive drugs
✓ Endocrine factors	Azathioprine
✓ Amenorrhea	Cyclophosphamide
✓ Premature menopause	Cyclosporine
✓ Low plasma levels of	✓ Chronic anticoagulation
androgens	✓ Lack of sun exposure
✓ Hyperprolactinemia	
✓ Induction of cytokines	
bone resorption	

Patients with SLE and neurological involvement (epilepsy, stroke, etc.) comprise the group at highest risk of fractures as they are more prone to falls and to the adverse effects of antiepileptics[32].

Ankylosing Spondylitis (AS)

AS is part of the group of seronegative spondyloarthropathies (SpA), a group of inflammatory joint disorders that also includes psoriatic arthritis, inflammatory bowel disease and reactive arthritis. AS is the typical form of SpA with symptoms related to arthritis, enthesitis, sacroiliitis, among others. The location of the primary disease is believed to be enthesis, i.e. the area where the tendons and ligaments are inserted into the bone[4].

Axial involvement generates a negative bone impact and increases the risk of vertebral fracture due to continuous inflammation, which results in a decrease in bone mass and progressive ankylosis, together with bone proliferation[33].

Osteoporosis can occur due to physical inactivity, reduced mobility of the spine related to pain, stiffness and ankylosis, in addition to subclinical involvement of intestinal disease[34].

The prevalence of osteoporosis in AS is approximately 14-27% in the spine and 4-14% in the hip[35,36]. Using the visual and morphometric definitions of vertebral size, the prevalence of fractures in this group of patients is 10-30%[37,38].

Prieto et al demonstrated a strong association between AS and vertebral fractures. These patients have a 5-fold higher risk of fracture compared to a control group. In addition, they observed that

the first 2.5 years of evolution of the disease are critical because the peak fracture risk increases and is believed to be secondary to the inflammatory[33]. These data are similar to those reported by Van der Wijden et al, suggesting that fractures in AS are associated with exacerbations of the disease. In addition, they showed that vertebral fractures were more frequent in men[39].

It is important to emphasize that this group of patients has a high risk of vertebral fractures. However, they do not present an increased risk of non-vertebral fractures.

Psoriatic Arthritis (PA)

PAs are also part of seronegative spondyloarthropathies. Studies on bone involvement in these patients are scarce, probably because the frequency of the disease is lower than that of rheumatoid arthritis and systemic lupus erythematosus. However, a relationship between inflammatory markers (TNF, IL-6, interferon-gamma or IFN-γ) and loss of bone mass have been demonstrated[40].

Keller et al showed that the group of patients with severe PAs have lower bone mass loss values than those with mild to moderate PA, showing that inflammatory factors play a fundamental role in bone resorption[40].

Dreiher et al analyzed 7,939 patients with psoriasis. The prevalence of osteoporosis was significantly higher compared to the control group and more frequent in women. However, it was observed that in the male group osteoporosis was the result of systemic disease (PAs), whereas in women it was shown to be due to an estrogen deficiency. In addition, women tended to be diagnosed before men because, usually, women are routinely referred for bone evaluation[41].

Osteoarthrosis (OA)

Osteoarthrosis is a degenerative disease that usually occurs over 45 years and whose origin is multifactorial, with joint disease being more frequent[4].

Arthrosis, together with osteoporosis, represent the two etiologies that affect the bone and joint structure in the elderly population and in turn are the primary cause of the deterioration of their quality of life[42].

Yoshimura et al carried out a 10-year follow-up study in patients with lumbar osteoarthritis and osteoporosis. The cumulative incidence of lumbar

osteoarthritis in 10 years within the age range of 40-79 years was 25.8% in men and 45.2% in women. In turn, there was a significant relationship between the presence of lumbar osteoporosis and the incidence of lumbar osteoarthritis[42].

The inverse relationship of arthrosis and osteoporosis has been debated for years. Zhang et al demonstrated the mechanical and microstructural structures of the subchondral trabecular bone of postmenopausal women with arthrosis and osteoporosis, through the determination of fractional bone volume and bone mineral density. The group of patients with osteoarthritis showed a greater fractional bone volume compared to the group of patients with osteoporosis. However, the relationship with bone density was not significant[43].

Regarding the production of fractures in these patients, the causal factor is believed to be falls. A longitudinal global study of osteoporosis found that approximately 40% of the population studied had osteoarthritis, and the direct relationship with fractures was not significant after adjusting for incidental falls. However, the adjusted relative risk for osteoarthritis as a predictor of falls was 1.24 (95% CI, 1.22 - 1.26, p<0.0001). In addition, postmenopausal women with osteoarthritis were 25% more likely to fall than women without osteoarthritis[33].

Glucocorticoid-induced Osteoporosis (GIO)

GIO is the most common cause of secondary osteoporosis. Prolonged treatment with 2.5-5 mg daily of prednisolone has been shown to increase the risk of vertebral and hip fracture[16,44].

GIO most commonly affects the areas of trabecular bone (lumbar spine and proximal femur). Fractures can occur in approximately 30-50% of patients receiving corticosteroids on an extended basis[44].

Table 4 describes some risk factors for GIO.

Pathophysiology

Glucocorticoids have a direct and indirect effect on metabolism by blocking the actions of vitamin D and calcium absorption, leading to a decrease in serum calcium and an increase in parathormone (PTH) levels[32]. However, raised PTH does not fully account for bone loss from glucocorticoid exposure[45].

During the initial period of treatment with corticosteroids, bone resorption increases, resulting in a sudden decrease in bone density and increased risk of fracture[45].

The specific mechanism by which corticosteroids induce bone resorption is by activating the RANKL receptor and the macrophage colony stimulating factor (MCSF). These two components are a fundamental part of osteoclastogenesis, along with decreased OPG receptor[46].

As for bone formation, it is affected due to the inhibition of osteoblast precursor cells and an increase in osteoblastic apoptosis[45]. In addition, there is inhibition of the function of mature osteoblasts and suppression of growth factors (IGF1) in bone cells, together with increased apoptosis of osteocytes[47,48] (Table 5).

Evaluation

Patients with osteoporosis should be investigated for possible causes of bone loss (Table 6). During physical examination, it is important to measure the patient's height and compare with previous measurements, in order to evaluate possible asymptomatic vertebral fractures.

Treatment

It is common to find suboptimal levels of vitamin D and calcium in these patients. Because of this, a correction treatment of these levels should be implemented.

In patients who begin treatment with glucocorticoids for more than 3 months at doses >5 mg/day, it is necessary to establish preventive treatment for bone mass loss, and in those patients who are already being treated, a bone densitometry should be carried out to assess the reduction of bone mass and the possible risk of fracture[17,49].

Due to increased use of corticosteroids and the prevalence of secondary osteoporosis, several international societies have described treatment guidelines. However, there is no global consensus on this matter[50-53]. In 2010 the ACR (American College of Rheumatology) published guidelines for the management of GIO, according to FRAX® (Figures 2 and 3)[54].

Non-pharmacological treatment

The management and prevention of GIO in non-pharmacological treatment is similar to that of primary osteoporosis and consists in eliminating modifiable risk factors[55]:
- Smoking.
- Alcohol consumption (≥3 unit/day).
- Sedentarism (energy consumption ≤1,682 kcal/day).
- Diet high in sodium (mainly in the presence of hypercalciuria).
- Decreased body mass index.

Patients with advanced age require special help to avoid falls, which are the main risk factor for fractures.

Calcium

Calcium and vitamin D are nutritional elements considered essential in any therapeutic option for osteoporosis.

The use of calcium carbonate at a dose of 1,000 mg/day has not been shown to prevent bone loss or reduce the risk of fracture in patients initiating prolonged corticosteroid therapy, particularly in postmenopausal women. Because of this, it is not indicated for primary prevention[56]; however, secondary prevention has shown that BMD levels remain constant in the lumbar spine in postmenopausal women with the use of 500 mg/day of calcium carbonate accompanied by 0.25 µg/day of calcitriol[55].

Table 4. Risk factors for glucocorticoid-induced osteoporosis

✓ Personal history of fracture
✓ History of fracture in first degree relative
✓ Current smoker
✓ Low weight (<57 kg)
✓ Advanced age
✓ Early menopause
✓ Low calcium intake
✓ Inadequate physical activity
✓ Alcoholism
✓ Recent falls
✓ Dementia
✓ Visual impairment
✓ Poor health status: chronic disease
✓ Decreased bone density levels

Table 5. Osteoporosis by glucocorticoids: effects on the bone

Osteoblasts	✓ Decreased osteoblastogenesis ✓ Increased apoptosis ✓ Continuous and early reduction of: 　　Trabecular osteoblasts 　　Synthetic capacity 　　Bone formation
Osteocytes	✓ Increased apoptosis ✓ Decreased canalicular circulation ✓ Decreased bone quality ✓ Osteonecrosis
Osteoclasts	✓ Increased osteoclastogenesis ✓ Transient and early increase of: 　　Survival of osteoclasts 　　Trabecular osteoclasts 　　Bone resorption

Table 6. Evaluation of secondary osteoporosis

Initial evaluation
✓ Detailed history for determination of possible risk factors
✓ Nutrition status assessment
✓ DMO
✓ Lumbar and thoracic spine (if there is loss of stature >1,5 in (≈3,8 cm)
✓ Hematologic biometry
✓ Calcium, phosphorus, 25(OH)-vitamin D, albumin and serum creatinine
✓ FSH, LH and prolactin
✓ Resorption and bone formation markers

Vitamin D

Active forms of vitamin D (alphacalcidol and calcitriol) and non-active forms (cholecalciferol and ergocalciferol) prevent the loss of bone mass in chronic glucocorticoid users[55].

Calciferol use prevents the reduction of bone mass, but does not reduce the incidence of fractures[57]. In contrast to non-active forms, the use of alfacalcidol at a dose of 0.25-1.0 µm/day plus 500 mg/day of calcium prevents the reduction of bone mass and reduces the risk of fracture (vertebral and non-vertebral)[58].

The combination of calcium and alphacalcidol has been the only one that has demonstrated a significant reduction in the risk of vertebral fracture. However, it shows no effect on non-vertebral fractures[58].

Randomized studies have shown that a dose of 700-800 IU of vitamin D reduces the risk of hip fracture and non-vertebral fractures in elderly patients[59]. For adults over 50, the National Osteoporosis Foundation recommends 800-1,000 IU of vitamin D per day. However, some experts recommend 1,000-2,000 IU daily, with a safety limit of 4,000 IU/day[60].

Bisphosphonates

The use of bisphosphonates has shown a positive effect on the loss of bone mass in patients who have been treated with glucocorticoids for prolonged periods[55].

In 3-to-5-year observational controlled studies, bisphosphonates have been shown to reduce vertebral and non-vertebral fractures including hip fracture[61]. In a meta-analysis carried out by Kanis et al, a significant reduction of non-vertebral fractures was demonstrated in comparison with the control group[62].

The use of bisphosphonates is recommended during the first two years of GIO, but there is insufficient evidence for long-term treatment[61].

In recent years, bisphosphonates have been the drugs of choice most commonly used to treat osteoporosis. Approved bisphosphonates for the treatment of glucocorticoid-induced osteoporosis are: etidronate, alendronate, risedronate and zoledronic acid. Contraindications to therapy include hypersensitivity or hypocalcemia; (≤30 ml/min of glomerular filtration rate for risedronate or ibandronate and ≤35 ml/min for alendronate and zoledronate) should be managed under surveillance[63].

Figure 2. Management of patients of both genders> 50 years who are initiating or receiving glucocorticoid (GC) therapy
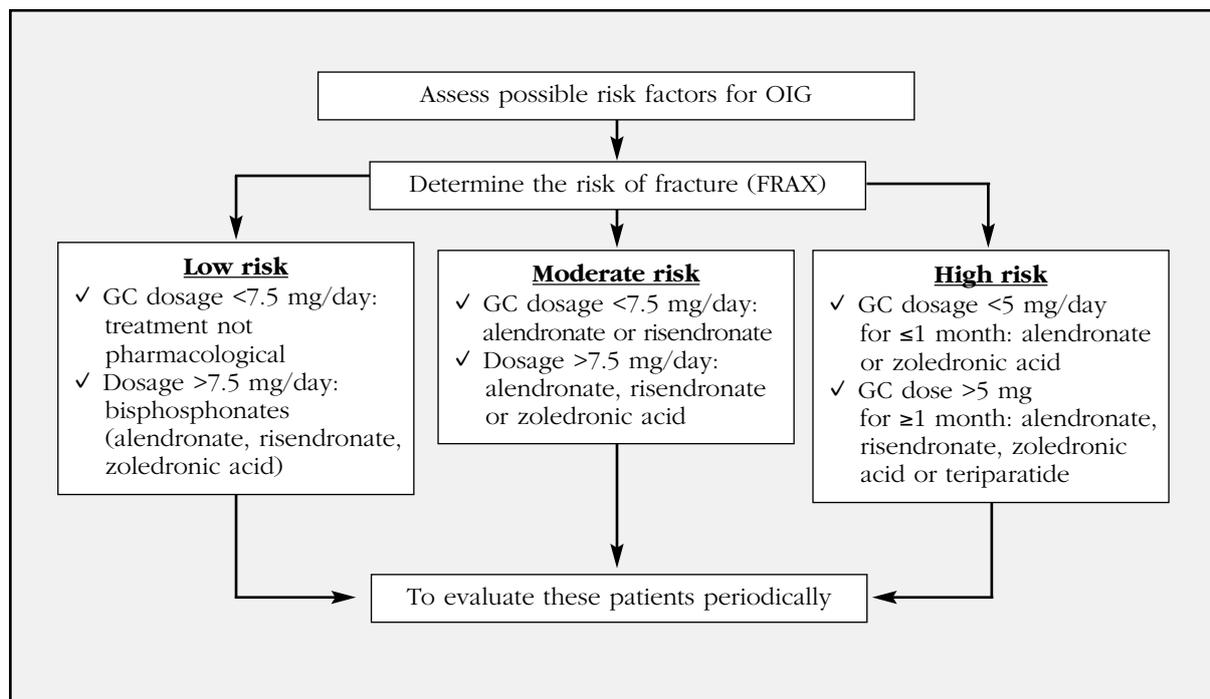
The use of alendronate 5-10 mg/day for 48 weeks has been shown to increase bone mass[64,65]. A study by Adachi et al demonstrated an increase in lumbar spine bone mineral density by 2.8% (5 mg/day) and 3.9% (10 mg/day) in patients with prolonged glucocorticoid therapy[66]. A dose of 5 mg/day of risedronate increases bone mass and also reduces the risk of fracture[67].

Zoledronic acid is approved by the Food and Drug Administration for the treating and preventing osteoporosis in men and postmenopausal women as well as glucocorticoid-induced osteoporosis. The appropriate dose of zoledronic acid is 5 mg intravenously infused once a year, which has been shown to reduce the risk of spine fracture, non-vertebral fracture and hip fracture in postmenopausal women with osteoporosis[68].

Teriparatide
Teriparatide is an analog of PTH obtained by the recombinant DNA technique (PTH-1-34). This analogous agent increases osteoblastic function and decreases the apoptosis of osteocytes[55].

The use of teriparatide at a dose of 20 mg/day subcutaneously should be considered as a treatment for GIO because it significantly increases bone mineral density in this group of patients, in addition to reducing vertebral fractures. However, it has no effect on non-vertebral fractures[69].

Because of the high cost of teriparatide, its use is recommended when bisphosphonates fail[69], that is, when despite treatment with bisphosphonates, the bone mass continues to decrease, against fractures in the presence of bisphosphonates, or if they are contraindicated[55].

Denosumab
Denosumab is an antibody against RANKL, which is used to treat primary osteoporosis. However, the study by Dore et al in patients with rheumatoid arthritis under treatment with GC, demonstrated an increase in BMD and reduced resorption, compared to placebo[70].

As denosumab is not filtered by the kidneys, it may be a therapeutic option for patients with renal dysfunction who do not tolerate bisphosphonates[70].

The use of denosumab for the treatment of osteoporosis induced by GC has not been approved yet, and its study is in stage III71. More evidence is needed to prove its utility in GIO[61].

Odanacatib
Odanacatib is a protease cathepsin-K inhibitor which induces bone deterioration by the osteoclast[72]. A phase II study by Bone et al, using odanacatib 50 mg once weekly, demonstrated an increase in bone mineral density in the lumbar spine (5.5% vs. 0.2% in the control group) and hip 5.5% (3.2% vs 0.9% in the control group)[73]. However, there are not enough controlled studies to determine the proper use of this drug in GIO, in addition to not being approved by the FDA so far.

Sclerostin Inhibitors
The mechanism responsible for the inhibition of bone formation in patients with prolonged glucocorticoid therapy is the reduction of the half-life and activity of osteoclasts. Glucocorticoids have been shown to alter bone cell formation by reducing the proliferation of osteoblasts and suppressing growth factors[49]. Because of this, new thera-

Figura 3. Management of patients of both sexes <50 years who are initiating or receiving glucocorticoid therapy (GC)
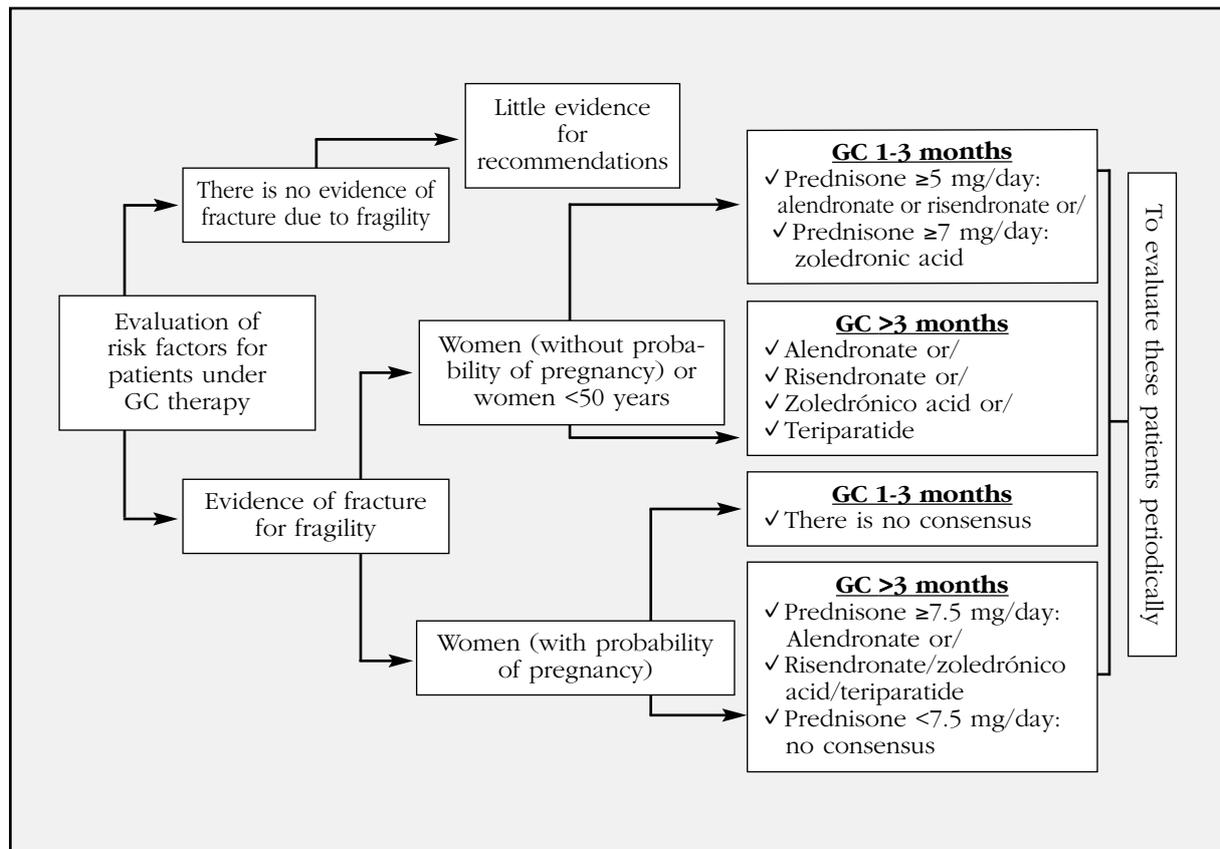
peutic approaches are based on maintaining the viability of osteoblasts and osteocytes in the presence of glucocorticoids.

Sclerostin (Sost) is a protein produced by osteocytes and its main function is to inhibit the maturation of osteoblasts[74-76]. A study by Yao et al demonstrated an increase in sclerostin gene expression in patients exposed to GC therapy for 28 days[77].

Monoclonal antibodies to sclerostin (Scl-Ab) currently known as romosozumab (AMG 785), blosozumab and BPS804 have been developed which inhibit the activity of sclerostin and thus stimulate bone formation[76,78].

Yao et al used Scl-Ab in mice exposed to 4 mg/kg/day of methylpredonoxone, and showed bone volumes of trabecular bone (Tb-BV/TV) in lower lumbar spine and neck of femur, less mass cortical bone in the middle third of the femur and lower cortical bone resistance compared to the placebo group. In addition, models receiving 25 mg/kg of Scl-Ab had a 60-125% increase in Tb-BV/TV and an increase in vertebral resistance of 30-70%[79].

Studies to date have shown that sclerostin inhibitors may be a future therapeutic option for managing patients with severe osteoporosis[80].

Conclusion

Osteoporosis is a systemic skeletal disease characterized by decreased bone mineral density with alterations in bone microarchitecture and increased risk of fracture.

Glucocorticoids are the primary cause of secondary osteoporosis, being an independent factor of morbidity and mortality in these patients, because the progressive loss of bone mass and increased risk of fracture begins shortly after the start of treatment with glucocorticoids.

It is important to identify, and, if possible, to correct risk factors and comorbidities in this group of patients, initiate preventive measures and provide health promotion tips such as change of habits, and give calcium and vitamin D supplements, in addition to treatment specific.

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Bibliography

1. Khosla S, Luftkin E, Hodgson S, Fitzpatrick L, Melton LJ 3rd. Epidemiology and clinical features of osteoporosis in young individuals. Bone. 1994;15(5):551-5.
2. Walsh L, Wong C, Pringle M, Tattersfield A. Use of oral corticosteroids in the community and the prevention of secondary osteoporosis: a cross sectional study. BMJ. 1996;313:344-6.
3. Sinigaglia L. Epidemiology of osteoporosis in rheumatic diseases. Rheum Dis Clin North Am. 2006;631-58.
4. Firestein G, Budd R, Gabriel S, McIness I, O'Dell J. Kelley's Textbook of Rheumatology. 9th ed. Elsevier; 2013. 2292 p.
5. Baker-LePain J, Nakumura M, Lane N. Effects of Inflammation on bone: an update. Curr Opin Rheumatol. 2011;23(4):389-95.

6. Walsh N, Crotti T, Goldring S, Gravallese EM. Rheumatic diseases: the effect of inflammation on bone. Immunol Rev. 2005;208:228-51.

7. Rifas L, Weitzmann M. A novel T cell cytokine, secreted osteoclastogenic factor of activated T cells, induces osteoclast formation in a RANKL-independent manner. Arthritis Rheum. 2009;60:3324-35.

8. Kong Y, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature. 1999;402:304-9.

9. Jódar Gimeno E. Conclusiones consensuadas del I Foro Multidisciplinar en el Manejo del Paciente con Alto Riesgo de Fractura (ARF) Osteoporótica. Rev Osteoporos Metab Miner. 2010;2(2):79-86.

10. Moon SJ, Ahn IE, Kwok SK, Park KS, Min JK, Park SH, et al. Periarticular osteoporosis is a prominent feature in early rheumatoid arthritis: estimation using shaft to periarticular bone mineral density ratio. Korean Med Sci. 2013;28:287-94.

11. Forsblad D, Larsen A, Waltbrand E, Kvist G, Mellstrom D, Saxne T, et al. Radiographic joint destruction in postmenopausal rheumatoid arthritis is strongly associated with generalised osteoporosis. Ann Rheum Dis. 2003;62:617-623.

12. Van Staa T, Geusens P, Bijlsma J, Leufkens H, Cooper C. Clinical assessment of the long-term risk of fracture in patients with rheumatoid arthritis. Arthritis Rheum. 2006;54(10):3104-12.

13. Edwards CJ, Williams E. The role of interleukin-6 in rheumatoid arthritis-associated osteoporosis. Osteoporos Int. 2010;21(8):1287-93.

14. Sinigaglia L, Nevertti A, Mela Q. A multicenter cross-sectional study on bone mineral density in rheumatoid arthritis. J Rheumatol. 2000;27:2528-9.

15. Stafford L, Bleasel J, Giles A. Androgen deficiency and bone mineral density in men with rheumatoid arthritis. J Rheumatol. 2000;27:2786-90.

16. Van Staa T, Leufkens H, Cooper C. The epidemiology of corticosteroid-induced osteoporosis: a meta-analysis. Osteoporos Int. 2002;13:777-87.

17. Deal C. Recent recommendations on steroid-induced osteoporosis: more targeted but more complicated. Clevel Clin J Med. 2013;80(2):117-25.

18. Di Munno O, Mazzantini M, Sinigaglia L, Bianchi G, Minisola G, Muratore M, et al. Effect of low-dose methotrexate on bone density in women with rheumatoid arthritis: results from a multicenter cross-sectional study. J Rheumatol. 2004;31:1305-9.

19. Seriolo B, Paolino S, Sulli A, Ferretti V, Cutolo M. Bone metabolism changes during anti-TNF-alpha therapy in patients with active rheumatoid arthritis. Ann NY Acad Sci. 2006;1069:420-7.

20. Marotte H, Pallot-Prades B, Grange L, Gaudin P, Alexandre C, Miossec P. A 1-year case–control study in patients with rheumatoid arthritis indicates prevention of loss of bone mineral density in both responders and non-responders to infliximab. Arthritis Res Ther. 2007;9(3):R61.

21. Lange U, Teichmann J, Müller-Ladner U, Strunk J. Increase in bone mineral density of patients with rheumatoid arthritis treated with anti-TNF-alpha antibody: a prospective open-label pilot study. Rheumatol. 2005;44:1546-8.

22. Van der Goes M, Jacobs G, Jurgens M, Bakker MF, van der Veen MJ, van der Werf JH, et al. Are changes in bone mineral density different between groupsof early rheumatoid arthritis patients treated according to a tight control strategy with or without prednisone if osteoporosis prophylaxis is applied? Osteoporos Int. 2013;24:1429-36.

23. Riggs B, Melton LJ 3rd. The worldwide problem of osteoporosis: insights afforded by epidemiology. Bone. 1995;115:505S-11S.

24. Mok C, Mak A, Ma K. Bone mineral density in post menopausal Chinese patients with Systemic Lupus Erythematosus. Lupus. 2005;14:106-12.

25. Lai CC, Chen WS, Chang DM, Tsao YP, Wu TH, Chou CT, et al. Increased serum fibroblast growth factor-23 and decreased bone turnover in patients with systemic lupus erythematosus under treatment with cyclosporine and steroid but not steroid only. Osteoporos Int. 2015;26(2):601-10.

26. Houssiau F, Lefebvre C, Depresseux G, Lambert M, Devogelaer JP, Nagant de Deuxchaisnes C. Trabecular and cortical bone loss in systemic lupus erythematosus. Br J Rheumatol. 1996;35:244-7.

27. Wang X, Yan S, Liu C, Xu Y, Wan L, Wang Y, et al. Fracture risk and bone mineral density levels in patients with systemic lupus erythematosus: a systematic review and meta-analysis. Osteoporos Int. 2016;4:1413-23.

28. Jacobs J, Korswagen LA, Schilder AM, Van Tuyl LH, Dijkmans BAC, Lems WF, et al. Six-year follow-up study of bone mineral density in patients with systemic lupus erythematosus. Osteoporos Int. 2013;24(6):1827-33.

29. Bultink E, Lems W, Kostense P, Dijkmans BA, Voskuyl AE. Prevalence and risk factors for low bone mineral density and vertebral fractures in patients with systemic lupus erythematosus. Arthritis Rheum. 2005;54:2044-50.

30. Toloza S, Cole D, Gladman D, Ibanez D, Urowitz M. Vitamin D insufficiency in a large female SLE cohort. Lupus. 2010;19:13-9.

31. Ruiz-Irastorza G, Egurbide M, Olivares N, Martinez-Berriotxoa A, Aguirre C. Vitamin D deficiency in systemic lupus erythematosus: prevalence, predictors and clinical consequences. Rheumatology. 2008;47:920-3.

32. Mazziotti G, Canalis E, Giustina A. Drug-induced osteoporosis: mechanisms and clinical implications. Am J Med. 2010;123:877-84.

33. Prieto-Alhambra D, Nogues X, Javaid K, Wyman A, Arden NK, Azagra R, et al. An increased rate of falling leads to a rise in fracture risk in postmenopausal women with self-reported osteoarthritis: a prospective multinational cohort study (GLOW). Ann Rheum Dis. 2013;72:911-7.

34. Ralston S, Urquhart G, Brezeski M, Sturrock RD. Prevalence of vertebral compression fractures due to osteoporosis in ankylosing spondylitis. BMJ. 1990;300:563-5.

35. Lange U, Kluge A, Strunk J, Teichmann J, Bachmann G. Ankylosing spondylitis and bone mineral density. What is the ideal tool for measurement? Rheumatol Int. 2005;26(2):115-20.

36. Cooper C, Carbone L, Michet C, Atkinson EJ, O'Fallon WM, Melton LJ 3rd. Fracture risk in patients with ankylosing spondylitis:a population based study. J Rheumatol. 1994;21:1877-82.

37. Mullaji A, Upadhyay S, Ho E. Bone mineral density in ankylosing spondylitis. DEXA comparison of control subjects with mild and advanced cases. J Bone Jt Sur Br. 1994;76:660-5.

38. Toussirot R, Michel F, Wendling D. Bone density, ultrasound measurements and body composition in early ankylosing spondylitis. Rheumatol. 2001;40:882-8.

39. van der Weijden M, van der Horst-Bruinsma I, van Denderen J, Dijkmans B, Heymans M, Lems W. High frequency of vertebral fractures in early spondylarthropathies. Osteoporos Int. 2012;23:1683-90.

40. Keller J, Kang J, Lin H. Association between osteoporosis and psoriasis: results from the Longitudinal Health Insurance Database in Taiwan. Osteoporos Int. 2013;24:1835-41.

41. Dreiher J, Weirzman D, Cohen A. Psoriasis and osteoporosis: a sex-specific association? J Inves Dermatol. 2009;129:1643-9.

42. Yoshimura N, Muraki S, Oka H, Mabuchi A, Kinoshita H, Yosihda M, et al. Epidemiology of lumbar osteoporosis and osteoarthritis and their causal relationship-is osteoarthritis a predictor for osteoporosis or vice versa?: the Miyama study. Osteoporos Int. 2009;20(6):999-1008.

43. Zhang ZM, Li ZC, Jiang LS, Jiang SD, Dai LY. Micro-CT and mechanical evaluation of subchondral trabecular bone structure between postmenopausal women with osteoarthritis and osteoporosis. Osteoporos Int. 2010;21(8):1383-90.

44. Pereira RM, Carvalho JF, Paula AP, Zerbini C, Domiciano DS, Gonçalves H, et al. Guidelines for the prevention and treatment of glucocorticoid-induced

osteoporosis. Rev Bras Reum. 2012;52(4):569-93.

45. Mirza F, Canalis E. Secondary osteoporosis: Pathophysiology and management. Eur J Endocrinol. 2015;173(3):R131-51.

46. Hofbauer L, Gori F, Riggs B, Lacey D, Dunstan C, Spelsberg T, et al. Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid- induced osteoporosis. Endocrinology. 1999;140:4382-9.

47. Dallas S, Prideaux M, Bonewald L. The osteocyte: an endocrine cell... and more. Endocr Rev. 2013;34:658-90.

48. Delany A, Durant D, Canalis E. Glucocorticoid suppression of IGF I transcription in osteoblasts. Mol Endocrinol. 2001;15(10):1781-9.

49. Weinstein R. Glucocorticoid-Induced Bone Disease. New Eng J Med. 2011;365:62-70.

50. American College of Rheumatology. Recommendations for the prevention and treatment of glucocorticoid-induced osteoporosis: 2001 update. American College of Rheumatology Ad Hoc Committee on Glucocorticoid-Induced Osteoporosis. Arthritis Rheum. 2001;44:1496-503.

51. Adler R, Hochberg M. Suggested guidelines for evaluation and treatment of glucocorticoid-induced osteoporosis for the Department of Veterans Affairs. Arch Intern Med. 2003;163(21):2619-24.

52. Devogelaer J, Goemaere S, Boonen S, Body J, Kaufman J, Reginster JY, et al. Evidence-based guidelines for the prevention and treatment of glucocorticoid-induced osteoporosis: a consensus document of the Belgian Bone Club. Osteoporos Int. 2006;17(1):8-19.

53. Messina O, Somma L, Tamborenea M, Castelli G, Riopedre A, Lancioni G, et al. Guías para el diagnóstico, la prevención y el tratamiento de la osteoporosis inducida por glucocorticoides en el adulto. Actual Osteol. 2016;12(2):107-25.

54. Grossman J, Gordon R, Ranganath V, Deal C, Caplan L, Chen W, et al. American College of Rheumatology 2010 recommendations for the prevention and treatment of glucocorticoid-induced osteoporosis. Arthritis Care Res. 2010;62(11):1515-26.

55. Rodrigues Pereira R, Freire de Carvalho J, Paula A, Zerbini C, Domiciano D, Gonçalves H, et al. Guidelines for the prevention and treatment of glucocorticoid-induced osteoporosis. Rev Bras Reum. 2012;52(4):569-93.

56. Sambrook P, Birmingham J, Kelly P, Kempler S, Nguyen T, Pocock N, et al. Prevention of corticosteroid osteoporosis. A comparison of calcium, calcitriol and calcitonin. N Eng J Med. 1993;328(24):1747-52.

57. Richy F, Ethgen O, Bruyere O, Regunster J. Efficacy of alfacalcidol and calcitriol in primary and corticosteroid-induced osteoporosis: a meta-analysis of their effects on bone mineral density and fracture rate. Osteoporos Int. 2004;14(5):301-10.

58. Ringe JD, Dorst A, Faber H, Schacht E, Rahlfs V. Superiority of alfacalcidol over plain vitamin D in the treatment of glucocorticoid- induced osteoporosis. Rheumatol Int. 2004;24(2):63-70.

59. Bischoff-Ferrari H, Willett W, Wong JB, Giovannucci E, Dietrich T, Dawson-Hughes B. Fracture prevention with vitamin D supplementation: a meta-analysis of randomized controlled trials. JAMA. 2005;293:2257-64.

60. Ross A, Manson J, Abrams S, Aloia JF, Brannon PM, Clinton SK, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab. 2011;96(1):53-8

61. Lems W, Saag K. Bisphosphonates and glucocorticoid-induced osteoporosis: cons. Endocrine. 2015;49(3):628-34.

62. Kanis JA, Stevenson M, McCloskey EV, Davis S, Lloyd-Jones M. Glucocorticoid-induced osteoporosis: a systematic review and cost-utility analysis. Health Technol Assess. 2007;11(7):1-231.

63. Kidney Disease: Improving Global Outcomes (KDIGO) CKD-MBD Work Group. KDIGO clinical practice guideli-

ne for the diagnosis, evaluation, prevention, and treatment of chronic kidney disease-mineral and bone disorders (CKD-MBD). Kidney Int Suppl. 2009;76(113):S1-130.

64. Saag K, Emkey R, Schnitzer T, Brown J, Hawkins F, Goemacre S, et al. Alendronate for the prevention and treatment of glucocorticoid- induced osteoporosis. N Eng J Med. 1998;339(5):292-9.

65. de Nijs R, Jacobs J, Lems W, Laan R, Algra A, Huisman A, et al. Alendronate or alfacalcidol in glucocorticoid-induced osteoporosis. N Engl J Med. 2006;355(7):675-84.

66. Adachi JD, Saag KG, Delmas PD, Liberman UA, Emkey RD, Seeman E, et al. Two-year effects of alendronate on bone mineral density and vertebral fracture in patients receiving glucocorticoids: a randomized, double-blind, placebo-controlled extension trial. Arthritis Rheum. 2001;44(1):202-11.

67. Cohen S, Levy R, Keller M, Boling E, Emkey R, Greenwald M, et al. Risedronate therapy prevents corticosteroid-induced bone loss: a twelve-month, multicenter, randomized, double-blind, placebo-controlled, parallel-group study. Arthritis Rheum. 1999;42(11):2309-18.

68. Watts N, Bilezikian J, Camacho P, Greenspan S, Harris S, Hodgson S, et al. American Association Of Clinical Endocrinologists Medical Guidelines for clinical practice for the diagnosis and treatment of postmenopausal osteoporosis: executive summary of recommendations. Endocr Pr. 2010;16(6):1016-9.

69. Saag K, Shane E, Boonen S, Marín F, Donley D, Taylor K, et al. Teriparatide or alendronate in glucocorticoid-induced osteoporosis. N Engl J Med. 2007;357(20):2028-39.

70. Dore RK, Cohen SB, Lane NE, Palmer W, Shergy W, Zhou L, et al. Effects of denosumab on bone mineral density and bone turnover in patients with rheumatoid arthritis receiving concurrent glucocorticoids or bisphosphonates. Ann Rheum Dis. 2010;69(5):872-5.

71. Whittier X, Saag K. Glucocorticoid-induced Osteoporosis. Rheum Dis Clin N Am. 2016;42(1):177-89.

72. Boonen S, Rosenberg F, Claessens F, Vanderschueren D, Papapoulos S. Inhibition of cathepsin K for treatment of osteoporosis. Curr Osteoporos Rep. 2012;10(1):73-9.

73. Bone H, McClung MR, Roux C, Recker RR, Eisman JA, Verbruggen N, et al. Odanacatib, a cathepsin K inhibitor for osteoporosis: a two-year study in post- menopausal women with low bone density. J Bone Miner Res. 2010;25:937-47.

74. Paszty C, Turner C, Robinson M. Sclerostin: a gem from the genome leads to bone-building antibodies. J Bone Min Res. 2010;25(9):1897-904.

75. van Bezooijen R, Roelen B, Visser A, van der Wee-Pals L, de Wilt E, Karperien M, et al. Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. J Exp Med. 2004;199(6):805-14.

76. Li X, Warmington K, Niu Q, Asuncion F, Barrero M, Grisanti M, et al. Inhibition of sclerostin by monoclonal antibody increases bone formation, bone mass and bone strength in aged male rats. J Bone Min Res. 2010;25(12):2647-56.

77. Yao W, Cheng Z, Busse C, Pham A, Nakamura M, Lane N. Glucocorticoid excess in mice results in early activation of osteoclastogenesis and adipogenesis and prolonged suppression of osteogenesis: a longitudinal study of gene expression in bone tissue from glucocorticoid-treated mice. Arthritis Rheum. 2008;58(6):1674-86.

78. McClung M, Grauer A, Boonen S, Bolognese M, Brown J, Diez-Perez A, et al. Romosozumab in postmenopausal women with low bone mineral density. New Eng J Med. 2014;370(5):412-20.

79. Yao W, Dai W, Jiang L, Lay EY, Zhong Z, Ritchie R, et al. Sclerostin-antibody treatment of glucocorticoid-induced osteoporosis maintained bone mass and strength. Osteoporos Int. 2016;27:283-94.

80. Appelman-Dijkstra N, Papapoulos S. Sclerostin inhibition in the management of osteoporosis. Calcif Tissue Int. 2016;98:370-80.
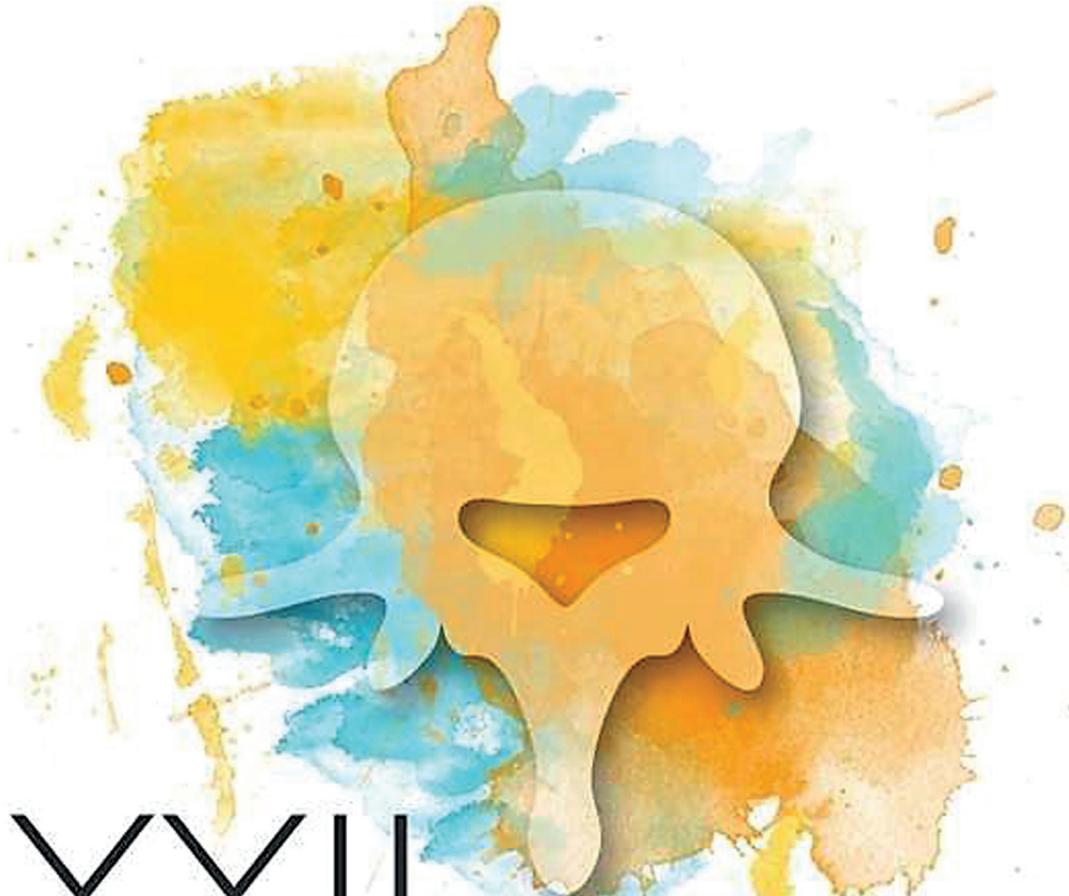
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