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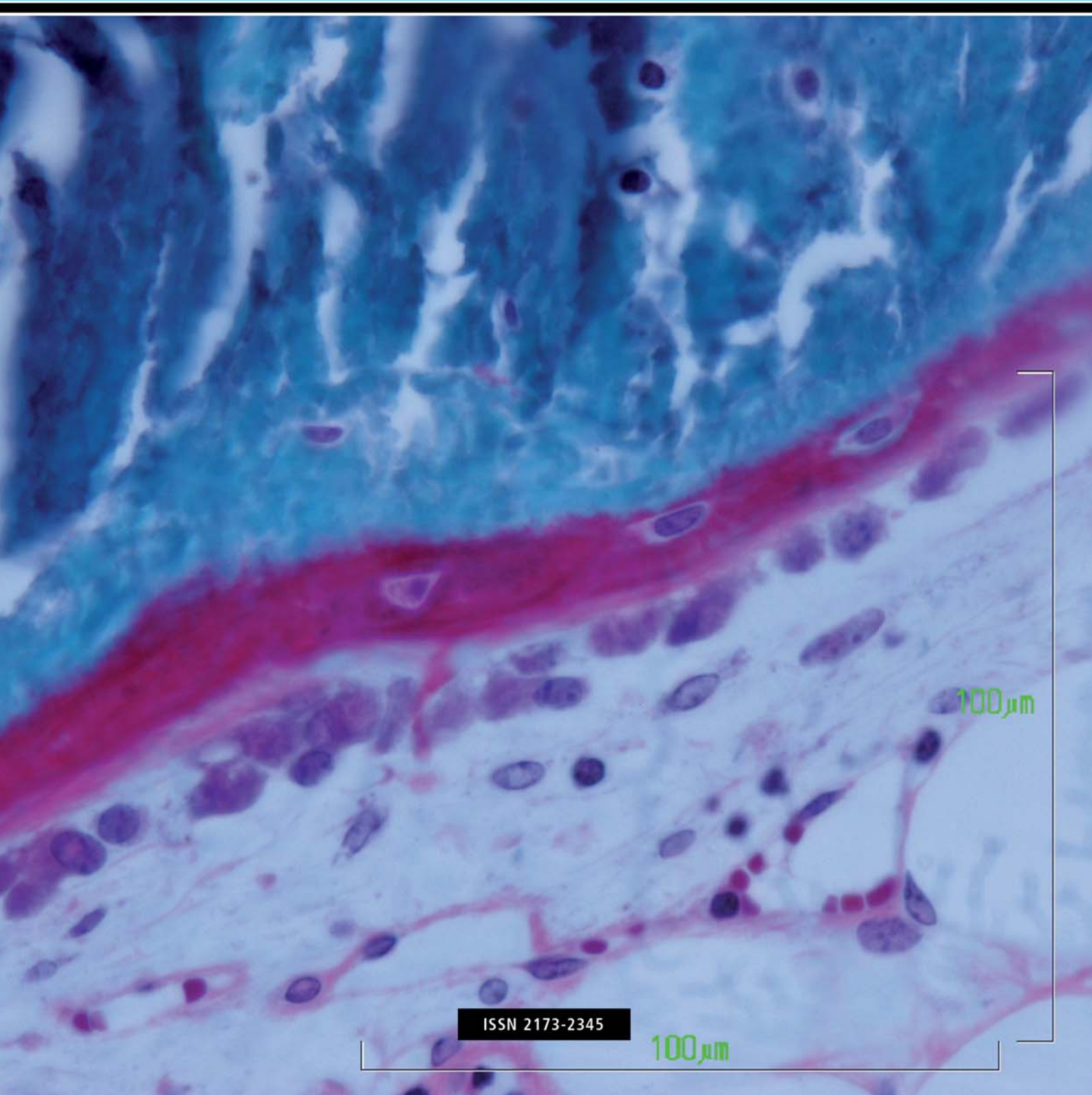

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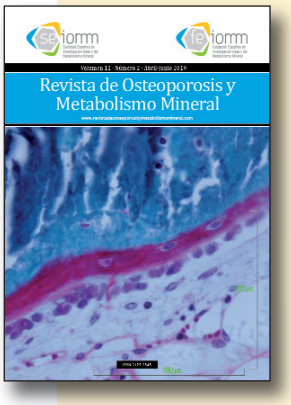

Our cover: Detail of osteoid edging on bone surface associated with a row of active osteoblasts. Immature osteocytes surrounded by non-mineralized bone matrix (red color) appear in the osteoid. Mature osteocytes can be seen in the mineralized bone matrix (blue color). Blood vessels are distinguished in the bone marrow (Staining: Goldner's Trichrome).
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Summary

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Editorial Committee

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Osteonecrosis of the jaw: lights and shadows in the knowledge of its pathophysiology

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Osteonecrosis of the jaw (ONJ) was described by Marx et al.[1] in 2005. In the following years, both isolated cases and series of patients were published which, over the years, was decreasing, on the one hand, due to the saturation of the journals and the low interest that the description of new cases may cause. Furthermore, knowledge of this disease has lead to the development of preventive measures that may have diminished its incidence.

Regarding ONJ, a whole range of "fears, risks and dangers" have been developed that are largely unjustified. ONJ was indicated as a complication of prolonged bisphosphonate treatment and in this sense it was equalized to the diaphyseal fractures[2], when both processes most certainly have different etiopathogenic mechanisms[3]. Fears concerning ONJ or diaphyseal fractures developed a whole doctrine about the need to suspend treatment with bisphosphonates or denosumab, the so-called "therapeutic vacations" that in reality what it was about was simply to suspend the antiresorptive treatment, before that the possible complications of its use appear[4-6]. This is especially common in the field of dentists, who, in many cases, concerned about the possible development of an ONJ do not perform virtually any dental intervention in patients receiving bisphosphonates or denosumab. With this, what has been observed is an increase in the abandonment of treatment with antiresorptive drugs which produces an increased risk of fragility fractures after discontinuation of bisphosphonate therapy, a risk that has an extreme severity in the case of suspension of denosumab treatment, with the appearance of multiple vertebral fractures[7-11].

ONJ occurs mainly in patients suffering from cancer (more than 90% of the cases described) and who have received potent bisphosphonates or denosumab at doses not used in osteoporosis treatment[3,12,13] and in which there has been a dental intervention[14]. Among patients receiving antiresorptives for the treatment of osteoporosis, the occurrence of cases, although it is true that it has been reported, is very scarce, almost exceptional[15,16].

In this issue of the Revista de Osteoporosis y Metabolismo Mineral, Quintana et al.[17] present the findings observed in a series of patients with ONJ in which they have carried out a complete study of both the amount of bone mass, determined by densitometry, and bone quality, estimated by the trabecular bone score (TBS) and Quantitative Ultrasound, an unfairly undervalued, harmless and simple technique that can assess bone quality and predict the risk of fracture as well as traditional densitometry[18,19]. The results obtained differ from the myths developed about ONJ and is that the excess suppression produced by these drugs would produce a "frozen" bone of poor quality and weakness. As can be seen in these results, it is most likely that the quantity and quality of the bone in ONJ does not show general alterations. Rather, involvement is local and influenced by multiple factors. All of this leads us to conclude that we still do not know many facts about the etiology, pathogenesis and pathophysiology of ONJ, and that we still have more shadows than lights on this matter[12,20,21].

Conflict of interests: The author declares no conflicts of interest.

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The Wnt/β-catenin pathway decreases the amount of osteoclasts in the bone and promotes its apoptosis

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Summary

The activation of Wnt/β-catenin signaling in cells of the osteoblastic lineage leads to an increase in bone mass through a dual mechanism: increasing osteoblastogenesis and decreasing osteoclastogenesis. The predominance of one mechanism over another depends on the maturational state of the osteoblast in which β-catenin accumulation occurs. The activation of Wnt/β-catenin signaling in cells of the osteoclastic lineage and its possible effects on the regulation of bone mass is less known. Previous studies have shown that conditional ablation of β-catenin in osteoclasts induces a decrease in bone mass associated with an increase in osteoclasts, and this fact has been attributed to an increase in osteoclastogenesis. However, other alternative possibilities have not been evaluated, such as that a decrease in the normal osteoclast apoptosis may also contribute to the greater number of osteoclasts. In this paper, to obtain information about this fact, we generated mice in which β-catenin was selectively eliminated from cells of the monocyte/macrophage lineage using an allele flanked by β-catenin (*Catnb^{f}*) together with the deletion line LisozimaMCre (*LysMCre*). The three-dimensional analysis of the bones of the *Catnb$^{f/f}$;LysM* mice revealed a significant decrease in the thickness of the femoral cortex, while the trabecular bone of the vertebrae was not affected. This phenotype was associated with a greater number of osteoclasts on the bone surface. The number of osteoclasts in the cultures from the *Catnb$^{f/f}$;LysM* mice was twice as high as in the cultures obtained from the control mice. The administration of WNT3a attenuated the osteoclast formation induced by M-CSF and RANKL *in vitro*. In addition, WNT3a promoted apoptosis of osteoclasts, and this effect was counteracted, both by the presence of DKK1 and by the absence of β-catenin. Taken together, these results support a cellular autonomous effect of β-catenin in the osteoclast, and provide convincing evidence of the proapoptotic role of β-catenin in these cells.

Key words: bone, osteoclasts, lysozyme M, β-catenin, Wnt, WNT3a.

Introduction

The accumulated evidence over the past few years has established that the Wnt/β-catenin pathway is crucial for bone formation and the maintenance of skeletal homeostasis[1,2]. Wnt proteins exert their cellular functions by activating different signaling pathways, commonly called canonical pathway and non-canonical pathways[3]. The former acts by controlling the amount of β-catenin not associated with cadherin, while the other routes do not require the presence of β-catenin[4]. At present, the signaling pathway mediated by β-catenin is the best studied and understood. Activation of the Wnt/β-catenin pathway begins at the cell membrane with the binding of certain Wnt ligands, such as Wnt3a, to the transmembrane receptors of the Frizzled family. This binding recruits the LRP5/6 co-receptor (low-density-lipoprotein receptor-related protein 5/6), to form a ternary complex that destabilizes a cytoplasmic conglomerate of proteins that would otherwise phosphorylate the β-catenin of the cytoplasm for its destruction in the proteasome[5-7]. So, after ligand binding to the receptor, β-catenin is not phosphorylated or destroyed, and, therefore, can accumulate in the cytoplasm, from where it will be transferred to the nucleus. There it joins the transcription factor TCF/LEF (T-cell factor/lymphoid enhancer factor) and induces target gene expression[8].

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The initial demonstration of the canonical Wnt pathway involvement in osteogenesis was provided by the identification of loss-of-function (LOF) and gain-of-function (GOF) mutations of the LRP5 co-receptor, responsible for the osteoporosis-pseudoglioma syndrome and of the hereditary phenotype of high bone mass, respectively[9,10]. The bone phenotypes of these mutations could be reproduced in mouse models with the function of the genetically modified LRP5/6 receptor. These studies revealed the osteo-anabolic role of Wnt/β-catenin signaling[11]. More recently, studies carried out with mice in which β-catenin activity has been manipulated in cells of osteoblastic lineage have established that β-catenin increases bone mass through different mechanisms depending on the stage of differentiation in which the osteoblastic cell is found[12-14]. Thus, the GOF of β-catenin exclusively in precursor cells stimulates its proliferation and maturation, but suppresses the alternative fate towards chondrocytic differentiation, thus producing an increase in bone mass[15]. Furthermore, when the GOF of β-catenin is produced in a later stage of the differentiation, a high bone mass is also achieved, but this occurs at indirect mechanism rates, that is, β-catenin induces the expression of osteoprotegerin (OPG) in the osteoblast, and OPG attenuates osteoclastogenesis[16,17].

The indirect participation of osteoclasts as mediators of some of the effects of Wnt/β-catenin signaling raised the question of whether this pathway could also have a direct role in the function of osteoclasts when activated in them. Otero et al.[18] and Albers et al.[19] eliminated β-catenin from osteoclast precursors using the mouse model LisozimaMCre (*LysMCre*), and found a decrease in bone mass in the trabecular compartment, with a parallel increase in the number of osteoclasts, thus showing that the β-catenin of osteoclasts is, in fact, involved in bone homeostasis. These authors, however, attributed the increased number of osteoclasts to an exclusive increase in osteoclastogenesis, without considering possible effects on apoptosis. We believe it is important to clarify this issue, since β-catenin is part of a signaling pathway that is currently considered an interesting therapeutic target. To obtain an idea of this problem, we have generated a similar mouse model, in which we have analyzed its phenotype, and also possible effects on osteoclast apoptosis.

Material and methods

1. Reagents

The WNT3a protein, the Dickkopf 1 protein (DKK1), the macrophage colony stimulating factor (M-CSF) and the soluble receptor activator of nuclear factor kappa-B ligand recombinant proteins (sRANKL) were purchased from R & D Systems (Minneapolis, USA).

2. Mutant mice

All procedures with animals were carried out in accordance with European Union standards and 3R principles. The experiments were reviewed and approved by the Bioethics Committee of the University of Cantabria. The mouse with the β-catenin allele flanked by the flox[20] sequence and the *LysMCre*[21] line have previously been described and obtained from the Jackson Laboratory. Mice were genotyped by PCR, using genomic DNA extracted from tail biopsy. The primers used for the detection of *LysMCre* were the following; foward GCGGTCTGGCAGTAAAAACTATC and reverse: GTGAAACAGCATTGCTGTCACTT, and the product size 102 bp. Reverse: CACCATGTCCTCTGTCTATTC, and the product size in this case would be 324 bp. The experimental mice were generated by a mating strategy consisting of two steps. The *LysMCre* heterozygous mice were crossed with mice whose *β-catenin* gene was flanked by flox sequences in homozygosis (*β-catenin$^{f/f}$*). To generate mice homozygous for the conditional allele of *β-catenin* with and without the Cre allele, the *β-catenin$^{f/+}$ mice;LysMCre* crossed with the *β-catenin$^{f/f}$* mice.

3. Micro-CT

A micro-CT analysis of the fifth lumbar vertebra was carried out after dissecting the bones, cleaning them, fixing them in ethanol, loading them in 12.3 mm diameter examination tubes and obtaining an image (μCT40, Scanco Medical, Basserdorf, Switzerland). The scans were integrated into 3-D voxel images (1,024 x 1,024 pixel matrices for each individual planar stack) and a Gaussian filter (sigma=0.8, support=1) was used to reduce signal noise. A threshold of 200 was applied to all the analyzed scans. The scans were performed at a medium resolution (E=55 kVp, I=145 μA, integration time=200 ms). The entire vertebral body was scanned with a transverse orientation that excluded pedicles and joint processes. The manual analysis excluded the cortical bone from the analysis. All trabecular measurements were made by manually drawing contours every 10 to 20 cuts and using the voxel count for bone volume per tissue volume and spherical filling distance transformation rates without assumptions about bone shape as a rod or plate. trabecular microarchitecture. The cortical thickness was measured in the middle of the femoral diaphysis.

4. Bone histology

The lumbar vertebrae (L1, L2 and L3) and the left femur were fixed in 4% paraformaldehyde overnight at 4ºC and decalcified for eight to nine days in 9% EDTA (pH 7.4) before inclusion in paraffin. The histomorphometric examination was performed on longitudinal sections of 7 μm of the femur for cortical bone and on the frontal sections of the vertebrae for spongy bone.

5. Cell cultures

To quantify the osteoclast progenitor cells, the bone marrow (BM) was purged from the long bones and seeded at a density of 50,000 cells/cm^2 in 48-well plates. After remaining 4-5 days in the culture dish with α-MEM medium (Invitrogen), supplemented with 10% FBS, 1% PSG, 30 ng/ml M-CSF and 30 ng/ml sRANKL (R & R). D Systems), the osteoclasts were fixed with 10% formalin for 15 min and stained for tartrate-resistant acid phosphatase (TRAP). Osteoclasts were quantified taking into account multinuclear cells and positive for TRAP staining. For the rest of the cultures, purified bone marrow cells were used, ie not adhered to the plate. Macrophages and osteoclasts were developed from bone marrow (BM) cells not adhered to the culture dish, and cultured for 4 days in the presence of M-CSF (130 ng/ml) to obtain macrophages, or for 4 days in the presence of M-CSF (30 ng/ml) and 30 ng/ml of RANKL to obtain osteoclasts.

6. Real-time quantitative PCR (Quantitative real-time PCR, qRT-PCR)

The total RNA was extracted with TRIzol reagents (Life Technologies). 1 μg of total RNA was used to produce first strand cDNA using the enzyme m-MLV RT (Invitrogen). The qRT-PCR was carried out using PreMix Ex taq

(Takara) and the data was analyzed using the Biorad software. The primers and probes for β-catenin [Mm01350385_g1 (fam)] and gapdh [Mm99999915_g1 (vic)] were manufactured by the TaqMan Gene Expression Assays service (Applied Biosystems). The relative levels of mRNA expression were normalized with the S2 ribosomal protein gene by the ΔCt method[22].

7. Analysis by Western blot

The protein levels of β-catenin and β-actin were analyzed using a mouse monoclonal antibody that recognizes β-catenin (BD Biosciences), and a mouse monoclonal antibody that recognizes β-actin (Sigma-Aldrich).

8. Analysis of apoptosis

Osteoclasts derived from OM cells not adhered to the culture dish extracted from *Catnb$^{f/f}$;LysMCre* mice and their controls were obtained. Once developed the osteoclasts in the culture dish were treated with WNT3a. After 24 hours of treatment, the cultures were fixed and stained by TUNEL and TRAP. The total number of osteoclasts and the number of apoptotic osteoclasts in each plate were quantified. They were considered apoptotic when at least one of their nuclei was TUNEL positive. The TUNEL method was carried out using the FragEL DNA fragmentation detection kit (EMD Chemicals, San Diego, California, USA) before staining for TRAP. Multinuclear TRAP positive and TUNEL positive cells were enumerated.

The activity of caspase-3 was measured by determining the degradation of the fluorometric substrate DEVD (Biomol Research Laboratories, Plymouth Meeting, Pennsylvania, USA), and the protein concentration was measured using a kit compatible with Bio-Rad detergent (Bio-Rad Laboratories, Hercules, California, USA).

9. Statistic analysis

All data are reported as the mean ± standard deviation. The mean values of each group were compared using the two-tailed Student's t-test.

Results

1. The specific elimination of β-catenin from osteoclast precursors

Conditional inactivation of *β-catenin (Catnb)* in cells of osteoclastic lineage was performed by crossing mice harboring a *β-catenin* floxeado allele (*Catnbf*)[20] with mice expressing the Cre recombinase enzyme under the control of the gene regulatory elements *Lysozyme* (*LysMCre*)[21]. This Cre line induces the recombination of the floxeado allele specifically in cells of monocyte-macrophage lineage and neutrophils. From this crossing, two cohorts (males and females) of animals were generated; an experimental cohort, with *Catnb$^{f/f}$;LysMCre* genotype and another control with *Catnb$^{f/f}$* genotype.

The *Catnb$^{f/f}$;LysMCre* mice were born in the expected Mendelian proportion, with similar body weight (Figure 1A) to the controls of the same litter and showed no evident phenotype. The cleavage of the *β-catenin* gene was confirmed by qRT-PCR. The *β-catenin* mRNA levels were analyzed in *ex vivo* cultures of bone marrow (BM) derived macrophages and osteoclasts. The macrophages and osteoclasts obtained from *Catnb$^{f/f}$;LysMCre* mice exhibited a 70% and 60% decrease in *β-catenin* expression, respectively (Figure 1B). We attribute the limited efficacy of recombination to the presence of cells from lineages other than myeloid in the culture plate that expressed normal levels of *β-catenin*. As expected, the expression level of β-catenin mRNA in kidney, liver and spleen was indistinguishable between the two genotypes (Figure 1C).

2. Animals lacking *β-catenin* in osteoclast precursors have a lower cortical bone thickness

At 28 weeks of age, a cohort of 15 animals was sacrificed by sex and genotype and the bone architecture of the fifth lumbar vertebra and the right femur was examined by micro-CT. The analysis revealed a reduced thickness of the cortical bone (Figure 2A), both in the males and in the females *Catnb$^{f/f}$;LysMCre*. However, the absence of β-catenin in the osteoclast precursors did not significantly alter the trabecular bone mass, neither in the vertebrae nor in the femur (Figure 2B). Neither the intertrabecular space, the trabecular thickness and the number of trabeculae (not shown) were affected. These results suggest that Wnt/β-catenin signaling in osteoclasts is important for the maintenance of cortical bone mass.

3. *Catnb$^{f/f}$;LysMCre* mice present more osteoclasts in the endosteum and more osteoclast progenitors in the bone marrow (BM)

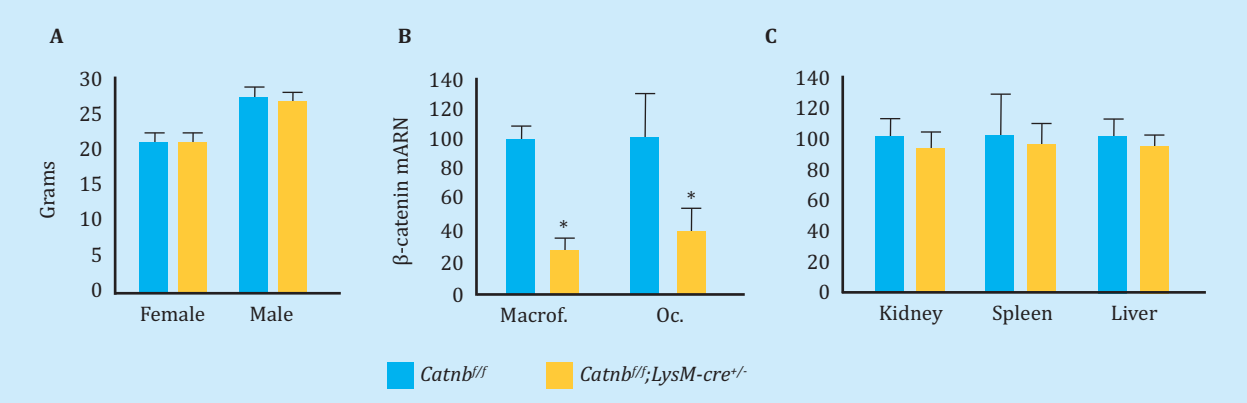
Next, we wanted to quantify the number of osteoclasts present on the surface of the cortical bone. What we found was that, according to a decreased cortical thickness, the number of osteoclasts on the endocortical surface of the femur of the *Catnb$^{f/f}$;LysMCre* mice was increased, as compared to the control animals (Figure 3A). The vertebral trabecular bone seemed to show the same tendency. However, the difference was not significant (p=0.06).

To examine whether the greater number of osteoclasts in bone could be explained by an increase in osteoclastogenesis, the number of these progenitors in MO was quantified. For this purpose, the MO cells obtained from the long bones of a 28-week-old mice were cultured in the presence of RANKL and M-CSF for 5 days. Three mice were used per genotype and determinations were made in triplicate for each of them. The number of osteoclasts that developed in cultures from *Catnb$^{f/f}$;LysMCre* mice showed a normal morphology (Figure 3B). However, the amount was twice as high as in the cultures from the control litter (426±18 per well vs 238±77, p=0.015) (Figure 3B). This result suggests that Wnt/β-catenin signaling in osteoclast precursors and their offspring attenuates the number of mature osteoclasts.

4. Proapoptotic effect of WNT3a requires the presence of β-catenin

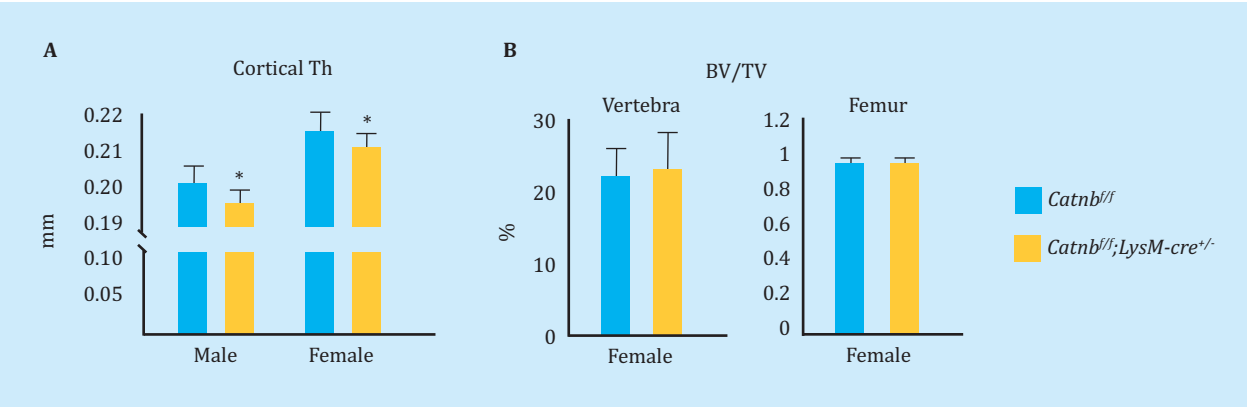
To examine the cellular mechanisms through which Wnt/β-catenin decreased the number of osteoclasts, we first determined whether the addition of WNT3a to the culture plate of the osteoclast precursors interfered with the development thereof. As shown in Figure 4A, the presence of WNT3a in the culture medium decreased the number of osteoclasts induced by the presence of M-CSF and RANKL. In addition, this fact seemed to be dose dependent. Western blot analysis confirmed an increase in β-catenin levels in osteoclasts exposed to WNT3a (Figure 4B). In addition, treatment of the culture with DKK1, an inhibitor of the LRP5/6 co-receptor, prevented the increase of β-catenin induced by WNT3a. The set of these findings indicates that WNT3a inhibits the development of osteoclasts by stimulating the canonical Wnt pathway.

Figure 1. *Catnb$^{f/f}$;LysM-cre$^{+/-}$* mice express lower levels of β-catenin in macrophages and osteoclasts. A: total body weight of two cohorts (15 animals per group) of mice *Catnb$^{f/f}$;LysM-cre$^{+/-}$* and their control litter *Catnb$^{f/f}$* of 28 weeks of age. B: quantitative analysis of mRNA by real-time PCR (Real Time-PCR) in macrophages and osteoclasts developed from non-adherent MO cells cultured in the presence of M-CSF for 4 days, and M-CSF plus RANKL for 5 days, respectively. C: quantitative analysis of soft tissue mRNA (indicated) obtained from 28-week-old mice (n=5)



Bars: values expressed as mean ± standard deviation; Oc: osteoclast; *p≤0.05.

Figure 2. *Catnb$^{f/f}$;LysM-cre$^{+/-}$* mice have lower cortical bone mass. Computed tomography (μ-CT) measurements carried out on the bones of 28-month-old mice (n=12-15 mice per group). A: cortical thickness (Cortical th) determined in femurs. B: BV/TV, bone volume per total tissue volume determined in L5 and right femur (only females are shown)



Bars: values expressed as mean ± standard deviation; Oc: osteoclast; *p=0.05.

The half-life of osteoclasts is known to be very short and they die by apoptosis. Next, we examined the effect of Wnt signaling on the apoptosis of macrophages and osteoclasts. As shown in figure 4C, apoptosis was determined by measuring the activity of caspase-3 after administration of increasing doses of WNT3A to the macrophage and osteoclast cultures. The results showed that WNT3a induced apoptosis in both macrophages and osteoclasts. The proapoptotic effect of WNT3 was also dependent on the dose used. The effect of WNT3a on osteoclast apoptosis was also analyzed by TUNEL (Figure 4D), and, in the same way as observed in the previous experiment, we saw that treatment with WNT3a increased the percentage of TUNEL positive osteoclasts. However, the presence of WNT3 had no deleterious effect on the osteoclast cultures obtained from *Catnb$^{f/f}$;LysMCre* mice (Figure 4D), lacking β-catenin. Likewise, the addition of DKK1 to the cultures abolished the proapoptotic actions of WNT3a (Figure 4D), indicating that the proapoptotic effect of WNT3a requires the presence of β-catenin. Taken together, these results support the hypothesis that the canonical signaling pathway of Wnt/β-catenin exerts proapoptotic effects on osteoclasts.

Discussion

In this study, we have analyzed the bone characteristics of animals lacking β-catenin in cells of the monocyte/macrophage lineage, which are the precursors of osteoclasts. These animals show a reduced cortical thickness associated with a greater number of osteoclasts on the surface of the endosteum and a greater number of osteoclast progenitors in the BM. In addition, we demonstrate that the stimulation of Wnt/β-catenin signaling in osteoclasts attenuates the amount of developed osteoclasts induced by the presence of M-SCF and RANKL, and promotes their apoptosis.

Otero et al.[18] and Albers et al.[19] used the same LysMCre line to eliminate *β-catenin* from osteoclast precursors. Both groups described a decrease in bone mass in the trabecular compartment, with a parallel increase in the number of osteoclasts, which they attributed to an exclusive increase in osteoclastogenesis. However, they did not address the possibility that a decrease in osteoclastic apoptosis was also implicated. In our study, we demonstrated an increase in osteoclasts on the endocortical surface of the femoral bone, and an increased number of

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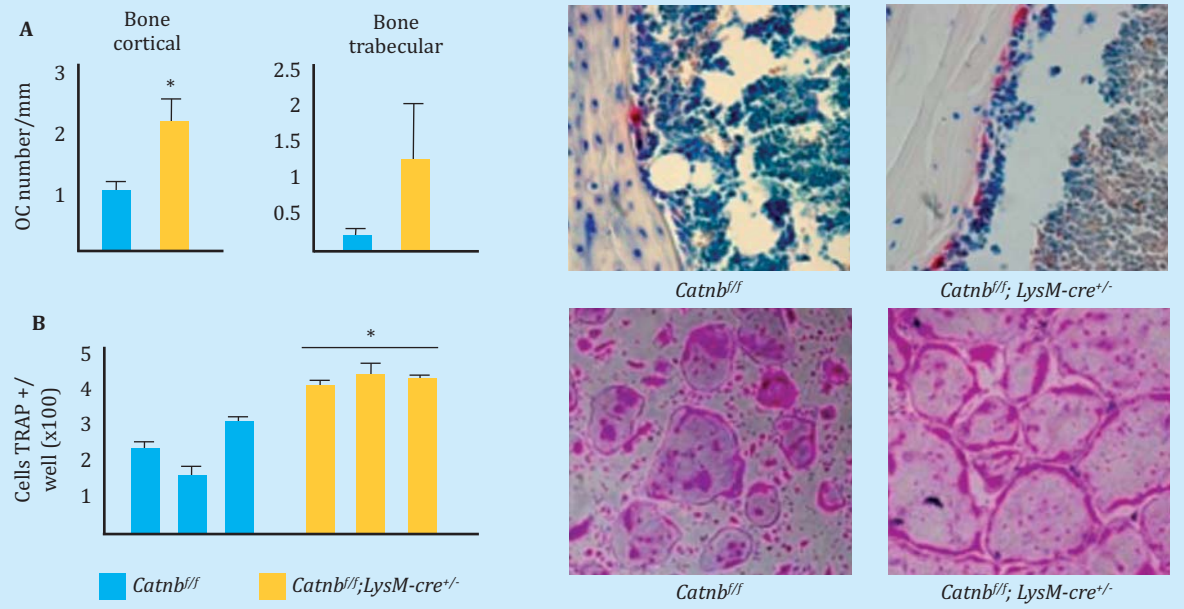
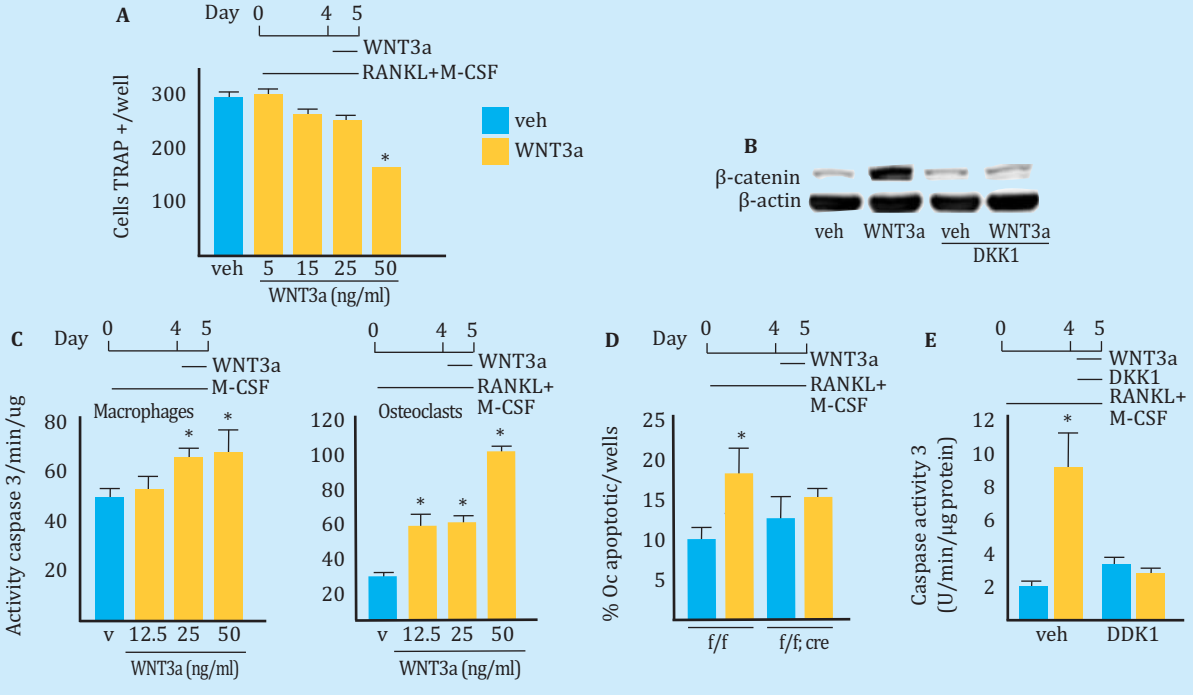
Figure 3. The *Catnb$^{f/f}$; LysM-cre$^{+/-}$* mice have more osteoclasts than the control litter. A: histomorphometric analysis of decalcified longitudinal sections of the femur and L1-L3 vertebrae of female mice aged 28 months (n=5 mice per group). Photomicrographs (x40) show representative areas of the endosteal bone surface after TRAP staining. B: number of TRAP-positive cells developed from MO cells, obtained from the femurs of 28-week-old mice, and cultured in the presence of M-CSF and RANKL for 5 days. Cultures were performed in triplicate of each of three animals separately. Each bar represents triplicates of each mouse, n=3 per group. Photomicrographs (x40) show representative areas of the culture plate



Bars: values expressed as mean ± standard deviation; Oc: osteoclast; *p=0.05.

Figure 4. WNT3a induces apoptosis of osteoclasts through the canonical Wnt pathway. A: number of TRAP-positive cells generated from MO cells not adhered to the plate obtained from C57BL/6 mice and cultured with M-CSF, RANKL and placebo (veh) or increasing doses of recombinant protein WNT3a as indicated. B: Western blot analysis of β-catenin in mature osteoclasts treated with veh, WNT3a, DKK1 or both. C: caspase 3 activity in mature macrophages and osteoclasts generated from non-adherent MO cells obtained from C57BL/6 mice and treated with veh or with different doses of WNT3a for 16 hours. D: TUNEL assay performed on mature osteoclast cultures generated from MO cells of *Catnb$^{f/f}$;LysM-cre* mice cultured with M-CSF and RANKL for 5 days and treated with veh or WNT3a (50 ng/ml) for 24 hours. hours. E: activity of caspase 3 in mature osteoclasts generated from nonadherent MO cells of C57BL/6 mice, and treated with veh, WNT3a (50 ng/ml) or DKK1 (1 µg/ml) for 24 h. AFU, arbitrary fluorescent units



Bars: values expressed as mean ± standard deviation; Oc: osteoclast; *p=0.05.

osteoclasts generated in cell cultures obtained from *Catnb$^{f/f}$;LysMCre* animals, compared to controls. We also observed that the stimulation of Wnt/β-catenin signaling in the MO cells obtained from wild mice decreases the number of osteoclasts developed in the culture plate. Our results, therefore, coincide with those of Otero et al.[18] and Albers et al.[19]. In addition, we have addressed the question of a possible involvement of osteoclastic apoptosis as part of the mechanism of action underlying the observed phenotypic findings. Our findings, in this sense, indicate that the decrease in the number of osteoclasts induced by the activation of Wnt/β-catenin is clearly due to the stimulation of apoptosis of macrophages and osteoclasts.

Several studies have shown that alterations in the survival of osteoclasts modify bone mass[23-26]. In fact, it is well established that estrogen protects the skeleton, in part, through proapoptotic effects on osteoclasts[27,28]. The elimination of estrogen receptor alpha in cells of the osteoclastic lineage, similar to the elimination of β-catenin, increases the number of osteoclasts and decreases bone mass. Unlike estrogen, glucocorticoids promote the loss of bone mass, at least in part, through the prolongation of the useful life of osteoclasts[29].

Wnts proteins have a positive effect on the survival of osteoblasts and also on osteoblastic progenitors that have not yet been compromised[30]. This antiapoptotic action of Wnts proteins on osteoblasts has been postulated as one of the mechanisms by which Wnt signaling increases bone mass[31]. Although the Wnt/β-catenin pathway is best known for its pro-survival effects, it can also exert proapoptotic actions. For example, the apoptosis of rat cardiomyoblasts induced by reoxygenation after hypoxia is regulated by WNT3a, through a mechanism dependent on β-catenin[32]. In addition, in line with these findings, Wnt/β-catenin signaling decreases the cellular invasiveness of melanoma[33], enhancing the expression of proapoptotic proteins, such as BIM and PUMA, and decreasing levels of antiapoptotic proteins, such as MCL[34].

In our study, through the analysis of caspase activity, or TUNEL assays, we have found that WNT3a induces osteoclast apoptosis. This effect is contrary to its previously mentioned pro-survival effect on osteoblasts[30]. Interestingly, like Wnts, estrogens and glucocorticoids exert opposite effects on the apoptosis of osteoblasts and osteoclasts[29,35,36].

To conclude, our findings suggest that the inhibitory effects of β-catenin on osteoclasts may be attributed to proapoptotic effects and support the claim that the osteoprotective effects of the canonical Wnt pathway also result from direct action through the osteoclastic lineage cells. Therefore, Wnt/β-catenin signaling in the bone environment has an osteoprotective effect exerted through both the osteoblastic and osteoclastic lineages.

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A novel therapeutic target for osteoarthritis: control of cellular plasticity and senescence using connexin43

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Summary

Introduction: Osteoarthritis (OA) is a degenerative musculoskeletal disease, which affects approximately the 13% of western population. Nowadays, there is no effective treatment for OA to avoid disease progression or to promote cartilage regeneration. Connexin43 (Cx43) is a transmembrane protein increased in cartilage and synovium from OA patients. Cx43 forms membrane channels that allow the exchange of molecules and ions between two adjacent cells through gap junctions (GJs), or between a cell and its environment through hemichannels. In this study we investigated the involvement of Cx43 and GJ intercellular communication in the degradation of articular cartilage in chondrocytes from patients with OA.

Material and methods: Primary chondrocytes were obtained from cartilage from OA and healthy donors. Protein levels were evaluated by western-blot, immunofluorescence and flow cytometry. RNA expression was evaluated by RT-qPCR. A scrape loading/dye transfer assay was used to evaluate cell communication. Cell senescence was analysed by flow cytometry or by light microscopy using β-galactosidase assay.

Results: Cx43 and GJs overactivities were correlated with the progression of OA, by promoting chronic cell dedifferentiation and senescence *in vitro* assays. We found that Cx43 overexpression activates factors involved in epithelial-to-mesenchymal transition, such as Twist-1. Increased levels of dedifferentiated cells, with high rates of cell proliferation, led to cell senescence via p53/p16^{INK4a}, activating the senescence-associated secretory phenotype (SASP) and promoting the synthesis and liberation of inflammatory factors, including the interleukin-6 (IL-6). Cx43 downregulation by using small molecules, such as oleuropein, or by genetic edition with CRISPR technology, led to the chondrocyte redifferentiation and an improved phenotype, with increased synthesis of extracellular matrix proteins such as Col2A1 and downregulating the synthesis of MMPs, inflammation and senescence.

Conclusions: Downregulation of Cx43 in OA chondrocytes restores regeneration by activating chondrocyte re-differentiation and decreasing cellular senescence. These results corroborate the use of Cx43 as an effective therapeutic target in order to restore cartilage regeneration and avoid OA progression.

Key words: connexin43, osteoarthritis, dedifferentiation, senescence, tissue regeneration.

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Introduction

Osteoarthritis (OA) is a chronic disease that is characterized by a progressive degradation of the articular cartilage that covers the surface of the synovial joints, which allow the movement of the skeleton without causing pain. Chondrocytes from patients with osteoarthritis undergo changes in the phenotype associated with an increase in catabolic and inflammatory activity[1,2], along with an increase in cellular senescence and senescence-associated secretory phenotype (SASP)[2,3]. Our research group has previously shown that chondrocytes in the articular cartilage have long cytoplasmic projections that cross the extracellular matrix (ECM)[4], which form connections and gap junctions (GJs) through connexin-43 channels (Cx43)[4,5]. In 2013, our research group published relevant results associated with alterations in the activity of Cx43 in osteoarthritis, indicating that from the disease's early stages there is an increase and changes in the localization of the protein in the cartilage of patients with arthrosis[6]. Subsequently, using animal models, we observed that the C-terminal domain of Cx43 plays a fundamental role in the structure and composition of articular cartilage[7].

Cx43 is involved in tissue regeneration processes in skin, heart and other tissues[8,9]. Several authors have reported that osteoarthritis could be included in diseases related to alterations in tissue regeneration[10,11]. In fact, arthritic chondrocytes undergo cellular dedifferentiation and present higher levels of cell proliferation[12,13], probably due to an attempt to repair the damage produced in the cartilage. The presence of chronically dedifferentiated chondrocytes triggers the progressive replacement of articular cartilage by fibrocartilage associated with degeneration and functional loss in the joint[14-19]. In this line of research, it is important to emphasize that the use of molecules that promote chondrogenesis, and therefore the re-differentiation of the chondrocyte, have a protective effect in OA[20] models. These molecules are called OA-modifying drugs (DMOADs), among which is kartogenin, which has been shown to promote chondrogenesis in human mesenchymal stem cells and also improve regeneration. of cartilage in mice subjected to inflammatory and/or mechanical damage in the joint[20]. Other DMOADs, such as TD-198946, TAK-778 or AG-041R, have also been described as promoter molecules of chondrogenesis with therapeutic potential in repair of articular cartilage[21-23]. The cartilage of patients with OA presents high levels of Cx43 together with alterations in the process of tissue regeneration. Our objective was to study whether alterations in Cx43 activity and intercellular communication through UCs would be related to changes in the cellular phenotype and senescence associated with disease progression.

Material and methods

Sample collection and cell culture

The cartilage samples were isolated and processed as previously described[4] after the donors signed the informed consent and the approval of the Clinical Research Ethics Committee of Galicia (C.0003333, 2012/094 and 2015/029) was granted. We used the human chondrocyte cell line T/C-28a2, from healthy primary chondrocytes that were transfected with the SV40 virus particle, donated by Dr. Mary Goldring (The Hospital for Special Surgery, New York, USA). The chondrocytes were cultured in DMEM medium (Dulbecco's modified Eagle's Medium, Lonza) supplemented with 10% fetal bovine serum (FBS) and a mixture of 1% antibiotics (P/S; Penicillin 100 U/mL, Streptomycin 100 µg/mL, Gibco).

Western blot

The analysis of total or nuclear protein levels was carried out using the Western blot technique. Equivalent amounts of proteins were separated in 10% denaturing acrylamide gels and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with skimmed milk diluted in tris buffered-saline (TBS), the membranes were incubated overnight at 4°C with anti-α-tubulin primary antibodies (Sigma-Aldrich, T9026), Cx43 (Sigma-Aldrich, C6129) Twist-1 (SCBT, sc-81417), cell proliferation nuclear antigen or PCNA (SCBT, sc-56), p53 (SCBT, sc-126), nuclear factor enhancer of the kappa light chains of activated B cells or NF-Kß (SCBT, sc-8008) or Lamin A (SCBT, sc-20680). After incubation with the primary antibody, the membranes were washed with TBS and incubated with their corresponding secondary antibodies labeled with horseradish peroxidase (HRP) for 1 hour at room temperature. Once the excess antibody was removed with TBS, the signal was visualized in a LAS-3000 development chamber (Fujifilm).

Immunofluorescence

For protein detection by immunofluorescence, cells previously fixed with 2% paraformaldehyde were incubated with 0.1 M glycine (Sigma-Aldrich) for 10 minutes. Subsequently, a permeabilization of the cell membranes was performed with Triton X-100 (Sigma-Aldrich) at 0.2% in phosphate buffered saline (PBS), for 10 minutes. Nonspecific junctions were blocked by a 30-minute incubation with 1% bovine albumin serum (BSA, Sigma-Aldrich) in PBS. Subsequently, the cells were incubated with the primary antibody for 1 hour at room temperature. After three 10-minute washes with PBS, the cells were incubated with the secondary antibody labeled with a fluorophore for 1 hour, in the dark and at room temperature. Three more 10-minute washes were carried out with PBS, followed by a staining of 4 'nuclei, 6-diamidino-2-phenylindol-DAPI- (Sigma-Aldrich). The images were made in an Olympus BX61 microscope with a DP71 camera.

Immunohistochemistry

Chondrocyte micromases were embedded in O.C.T.™ (Optimum Cutting Temperature) compound and cut into 4 µm sections, which were incubated with 3% hydrogen peroxide for 10 minutes. Sections were incubated with the primary anti-collagen type II antibody for 1 hour at room temperature. After three washes with PBS, the sections were incubated with OptiView HQ Universal Linker (Roche) for 10 minutes. Subsequently, they were incubated for 8 minutes with OptiView HRP Multimer (Roche), the excess reagent was washed and the signal was revealed in a solution of 0.1% DAB in 0.02% hydrogen peroxide.

Flow cytometry

For the measurement of protein levels by flow cytometry, the cells were fixed with 1% paraformaldehyde for 10 minutes, washed with a wash solution (PBS + 0.5% BSA + 2mM EDTA), and stained with antibodies anti-Cx43-APC (R & D Systems, FAB7737A), endoglin or CD105-PE (Immunostep, 105PE-100T) or CD166 antigen (ALCAM) or

CD166-APC (Immunostep, 1399990314). The analysis was carried out on a FACSCaliburTM cytometer.

Cell transfection

The T/C-28a2 cell line was transfected by electroporation with the Amaxa® Cell Line Kit Nucleofector® kit V (Lonza) in a Cell Line Nucleofector™ (Lonza). One million cells were electroporated with 3 µg of plasmid pIRESpuro2 (Clontech) containing the sequence of the human Cx43 gene, donated by Dr. Arantxa Tabernero (INCYL, University of Salamanca, Spain). At 24 hours the medium was changed by means of P/S and antibiotic for the selection of the chondrocytes containing the plasmid.

On the other hand, the electroporation of the T/C-28a2 line was also carried out with a CRISPR vector (modified from Addgene #48138) with the enzyme Cas9 VP12 (derived from Addgene #72247) bound to a GFP marker (green protein). fluorescent), with a guide that targets 20 nucleotides of the Cx43 gene. This vector has been donated by Dr. Trond Aasen (Vall d'Hebron Research Institute, Autonomous University of Barcelona, Spain). Electroporated and positive cells for GFP were seeded in a 96-well plate and expanded as clones.

Gene expression

Gene expression levels were carried out by extracting mRNA with TRIzol (Invitrogen), retrotranscription with SuperScript® VILO ™ kit (Invitrogen) and quantification by quantitative real-time PCR in a LightCycler®480 (Roche). Primers were used for:

– *hypoxanthine phosphoribosyltransferase-1 -HPRT-1-* (5'-TTGAGTTTGGAAACATCTGGAG-3 '; 5'-GCCCAAAGGGAACTGATAGTC-3'), - GJA1 (5'-ACATGGGTGACTGGAGCGCC-3 '; 5'-ATGATCTGCAGGACCCAGAA-3'),

– *interleukin-1β -IL-1β-* (5'-CGAATCTCCGACCACCACTAC-3 '; 5' - TCCATGGCCACAACAACTGA-3 '),

– *interleukin-6 -IL-6-* (5 '-TGTAGCCGCCCCACACA-3'; 5 '-GGATGTACCGAATTTGTTTGTA-3'),

– *prostaglandin-endoperoxide synthase-2 -PTGS2-* (5'-CTTCACGCATCAGTTTTTCAAG -3 '; 5'-TCACCGTAAATATGATTTAAGTCCAC-3'),

– *metalloprotease 3 -MMP-3-* (5'-CCCTGGGTCTCTTTCACTCA-3 '; 5'-GCTGACAGCATCAAAGGACA-3'),

– *cyclin-dependent kinase inhibitor-2 -CDKN2-* (5'-GAGCAGAACGATAGGGCTTG-3 '; 5'-CAT GTGCCCTCT CCTCCTAA-3').

CUs Activity

Cell communication through communicating junctions was evaluated using a Scrape Loading/Dye Transfer (SL/DT) test. For this, a cut is made on confluent cells with a scalpel and the tip of a needle in Lucifer Yellow fluorescent compound (LY, Cell Projects Ltd© Kent, UK), incubating at 37°C for 5 minutes. The damaged cells that manage to repair the membrane take the fluorescent compound from the medium. The transfer of LY from the cut line was evaluated in an inverted fluorescence microscope (Nikon Eclipse Ti) and the ratio between undamaged cells positive for LY was calculated between the number of cells taking the compound through a damage in the membrane.

Senescence

Cellular senescence was evaluated according to β-galactosidase activity with a commercial kit with X-gal as a substrate (Senescence Cells Histochemical Kit, Sigma-Aldrich) and also by flow cytometry with the substrate di-β-galactopyranoside, which results in green fluorescence when hydrolyzed (Invitrogen). In the case of X-gal, the cells with β-galactosidase activity will be stained greenish blue, so that they can be analyzed under a visible light microscope. On the other hand, the hydrolysis of the di-β-galactopyranoside substrate was detected on a FACSCalibur™ cytometer, and the mean fluorescence was normalized to the untreated cell levels.

Statistic analysis

The GraphPad Prism program (version 5.00) was used to analyze the data. Student's t or Mann-Whitney U were used to analyze quantitative variables. The statistically significant differences were considered before values of $p<0.05$.

Results

Cx43 activates the catabolic activity in chondrocytes of patients with OA

Concurring with what was observed in tissue[6], articular chondrocytes in primary culture from donors with OA (OAc) had significantly higher Cx43 levels than those isolated from healthy donors (N) detected by flow cytometry (Figure 1A). The high levels of Cx43 were correlated with higher levels of intercellular communication through UCs, quantified by an SL/DT transfer assay of LY (Figure 1B). In order to study the effect on the cellular phenotype of high levels of Cx43 and intercellular communication through UCs, a healthy donor chondrocyte cell line, T/C-28a2, was used as a study model. Cx43 was overexpressed using a vector with the human Cx43 gene under the CMV[24] promoter (Figure 1C). The increase in Cx43 in the human chondrocyte cell line T/C-28a2 was correlated with an increase in the activity of the UCs detected by the SL/DT assay (Figure 1D). The gene expression assay by RT-PCR showed a significant increase in the gene expression of interleukin 1-β (IL-1β), cyclooxygenase-2 (COX-2) and metalloprotease-3 (MMP-3) when the Cx43 was overexpressed in the healthy chondrocyte line (T/C - Cx43) (Figure 1E).

Activation of cell dedifferentiation in OA

Using flow cytometric assays, we studied the levels of cell de-differentiation markers in chondrocytes from patients with osteoarthritis and chondrocytes isolated from healthy donors, in order to confirm the presence of immature chondrocytes in cartilage samples from patients with OA. By flow cytometry, higher levels of the CD166 "Stem" marker were detected in OAc in primary culture compared to healthy chondrocytes (Figure 2A). Consistent with these results, the increase of Cx43 in healthy chondrocytes (cell line) using an expression vector (T/C-Cx43 or T/C-28a2 line transfected with a plasmid to overexpress Cx43) triggered a significant increase in the levels of the two "stem-like" markers CD166 and CD105, with respect to the control cells with low levels of Cx43 (T/C-28a2) (Figure 2B).

The decrease in the activity of the Cx43 and the UCs activates cellular re-differentiation in OA

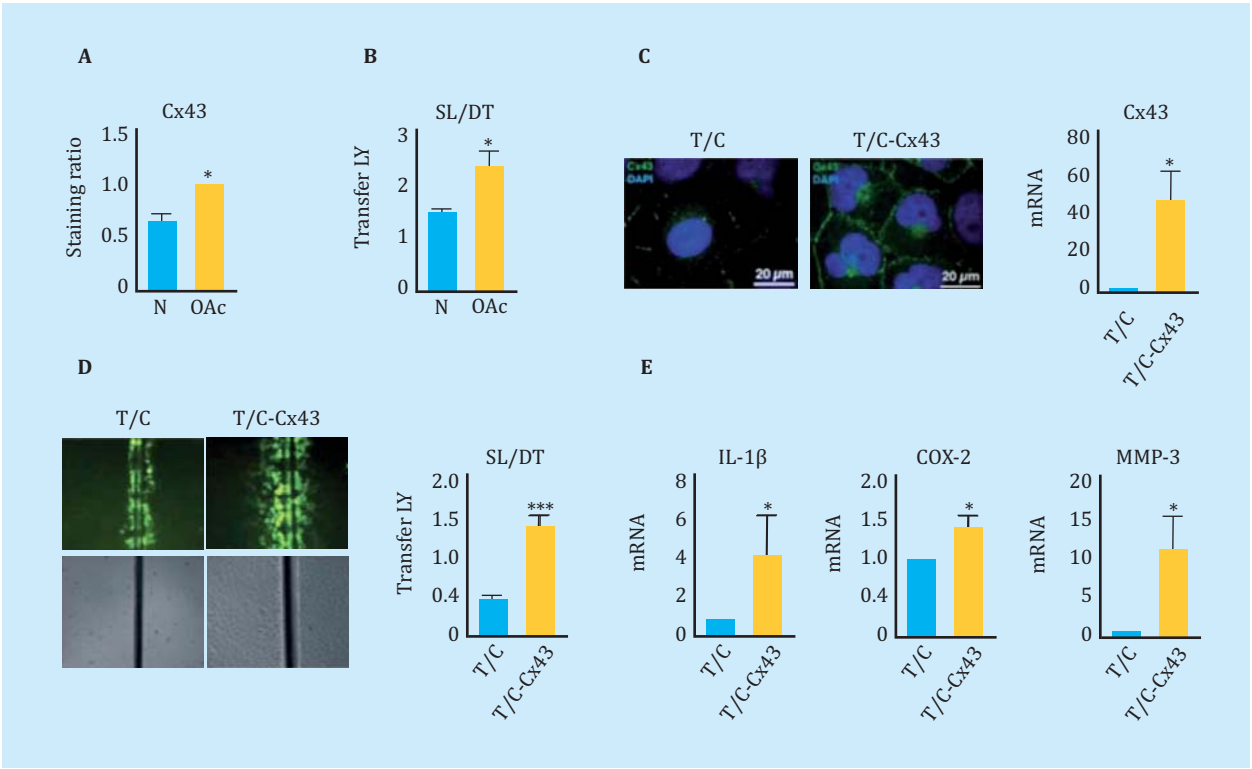
To reduce Cx43 activity in OAc, the effect of different molecules on the levels and activity of Cx43 was studied. In this study, we observed that the polyphenol

oleuropein decreases the levels of Cx43 in OAc (Figure 3A). The decrease in Cx43 levels improved the OA chondrocyte phenotype detected by an increase in the main marker of articular chondrocytes, collagen II (Figure 3B). The treatment of OAc with a concentration of 10 µM of oleuropein for 7 days significantly decreased levels of CD105 and CD166 dedifferentiation markers (Figure 3C), as well as the gene expression of IL-1β, IL-6, COX-2 and MMP-3 detected by flow cytometry and analysis of gene expression respectively (Figure 3D). The effect of Cx43 on cellular plasticity in OAc was confirmed in 3D culture. Modulation of Cx43 levels in the presence of 10 µM oleuropein in micromasses and in chondrogenic medium improved the structure of the extracellular matrix, detecting a significant increase in collagen II deposits and proteoglycans in the 3D structure matrix (Figure 4).

Cx43 activates TEM and cellular senescence in OAc

The overexpression of Cx43 in the line of chondrocytes T/C-28a2, was correlated with an increase in the nucleus of PCNA, protein used as a marker of cell proliferation, and with activation of the transcription factor related to TEM, Twist-1, detected by translocation and increased levels of the transcription factor at the nuclear level (Figure 5A). The transfected chondrocytes to overexpress the Cx43 also showed higher nuclear levels of NF-kß, one of the most important transcription factors in the regulation of synthesis of the SASP component (Figure 5A). Elevated levels of Cx43 correlated with elevated levels of factors involved in p53 cellular senescence (Figure 5B) and p16 (Figure 5C). OAc treatment with 10 µM oleuropein reduced Cx43 levels (Figure 3A) and cellular senescence detected by β-galactosidase activity by light microscopy and flow cytometry (Figure 5D). In order to confirm the effect of the Cx43 decrease in TEM and cellular senescence, the T/C-28a2 line was transfected with a CRISPR/Cas9 plasmid, obtaining heterozygous cells for the Cx43 gene (Figure 6A). Reduced levels of Cx43 on the T/C-28a2 line correlated with a significant decrease in the "stem-like" markers CD166 and CD105 (Figure 6B). The decrease in Cx43 levels in these cells, triggered a decrease in the levels of transcription factors Twist-1 (TEM) and NF-kβ (SASP) at the nuclear level (Figure 6C), decreasing the levels of cellular senescence , detected by β-galactosidase activity and flow cytometry (Figure 6D). T/C-28a2 chondrocytes with low levels of Cx43 (CRISPR-Cx43) showed significantly lower levels of synthesis of the pro-inflammatory mediators IL-1β and IL-6, and of protease MMP-3, with respect to the line T/C-28a2 without transfecting.

Figure 1. (A) Cx43 levels analyzed by flow cytometry comparing healthy (N) and osteoarthritic (OAc) human chondrocytes in monolayer culture. n=3, mean ± standard error of the mean (EEM); ***p<0.0001; Student t test. (B) Quantification of the Scrape Loading/Dye Transfer (SL/DT) cellular communication assay comparing chondrocytes from healthy (N) and osteoarthritic (OAc) donors. n=8, mean ± SEM; **p<0.01; Mann-Whitney test. (C) On the left, immunofluorescence for Cx43 (green) in chondrocytes T/C-28a2 (T/C) and the same line transfected with a plasmid to overexpress Cx43 (T/C-Cx43). The nuclei have been stained with DAPI (blue). On the right, gene expression levels of Cx43 in these two chondrocyte lines. n=5, mean ± SEM; *p<0.05; Mann-Whitney test. (D) Quantification of the SL/DT cellular communication assay, comparing the T/28a2 (T/C) line and transfected with a plasmid to overexpress the Cx43 (T/C-Cx43). n=10, mean ± SEM; ***p<0.0001; Mann-Whitney test. (E) Levels of gene expression of IL-1β, COX-2 and MMP-3 in the T/C-28a2 line that over-expresses Cx43 (T/C-Cx43) compared to the line transfected with a control plasmid (T/C). n=4, mean ± SEM; *p<0.05; Mann-Whitney test



Discussion

During osteoarthritis, the chondrocytes have increased levels of the transmembrane protein Cx43[6] and their phenotype is altered preventing them from participating in tissue regeneration and carrying out their function, triggering progressive tissue degeneration. The dedifferentiation related to epithelial-mesenchymal transition phenomena (TEM) is a cellular process that participates in the regeneration of tissues by allowing the cells to dedifferentiate into a more immature state to activate processes, including cell proliferation and migration, with the objective of replacing the damaged cells and remodeling the extracellular matrix[25,26]. However, when this dedifferentiation occurs chronically it can cause the development of fibrosis in the context of tissue regeneration[27,28]. In this study we have described that the levels of Cx43 and intercellular communication through UCs in osteoarthritis correlate positively with the cell de-differentiation markers CD105 and CD166. In addition, we have verified that this state can be partially reversed by the use of molecules that decrease the levels of Cx43, improving the phenotype of arthritic chondrocytes and

Figure 2. (A) Measurement of CD166 dedifferentiation marker by flow cytometry in arthritic chondrocytes (OAc) and chondrocytes from healthy donors (n=9, mean ± SEM, ***p<0.0001, Mann-Whitney test). (B) Levels of markers CD105 (n=5) and CD166 (n=7) measured by flow cytometry in the T/C-28a2 cell line that over-expresses Cx43 (T/C-Cx43) compared to the same line transfected with a control plasmid (T/C). Mean ± EEM; *p<0.05; Mann-Whitney test

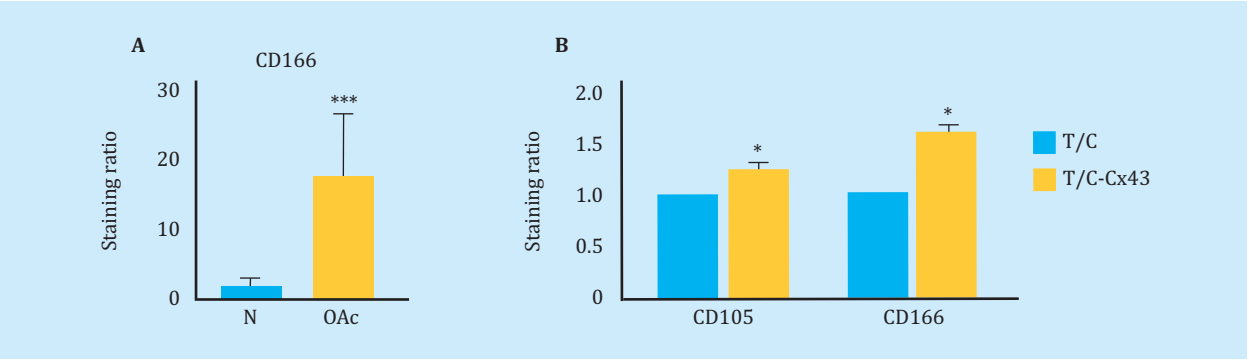


Figure 3. (A) Western blot to detect Cx43 in arthritic chondrocytes (OAc) in primary culture untreated (NT) or treated with 10 µM oleuropein (Oleu) for 2 hours. (B) Co-immunofluorescence of Cx43 (green) and collagen type II (red) of OAc treated with 10 µM oleuropein for 2 h. The cell nuclei appear in blue due to DAPI staining. The white arrows indicate Cx43 located in the cell membrane. (C) Levels of CD105 and CD166 markers measured by flow cytometry in OAc treated with 10 µM oleuropein for 7 days (n=5, mean ± SEM, **p<0.01, Mann-Whitney test). (D) Gene expression levels of IL-1β, IL-6, COX-2 and MMP-3 in OAc treated with 10 µM oleuropein for 2 hours (n=3-7, mean ± SEM; *p<0.05, ***p<0.0001, Mann-Whitney test)

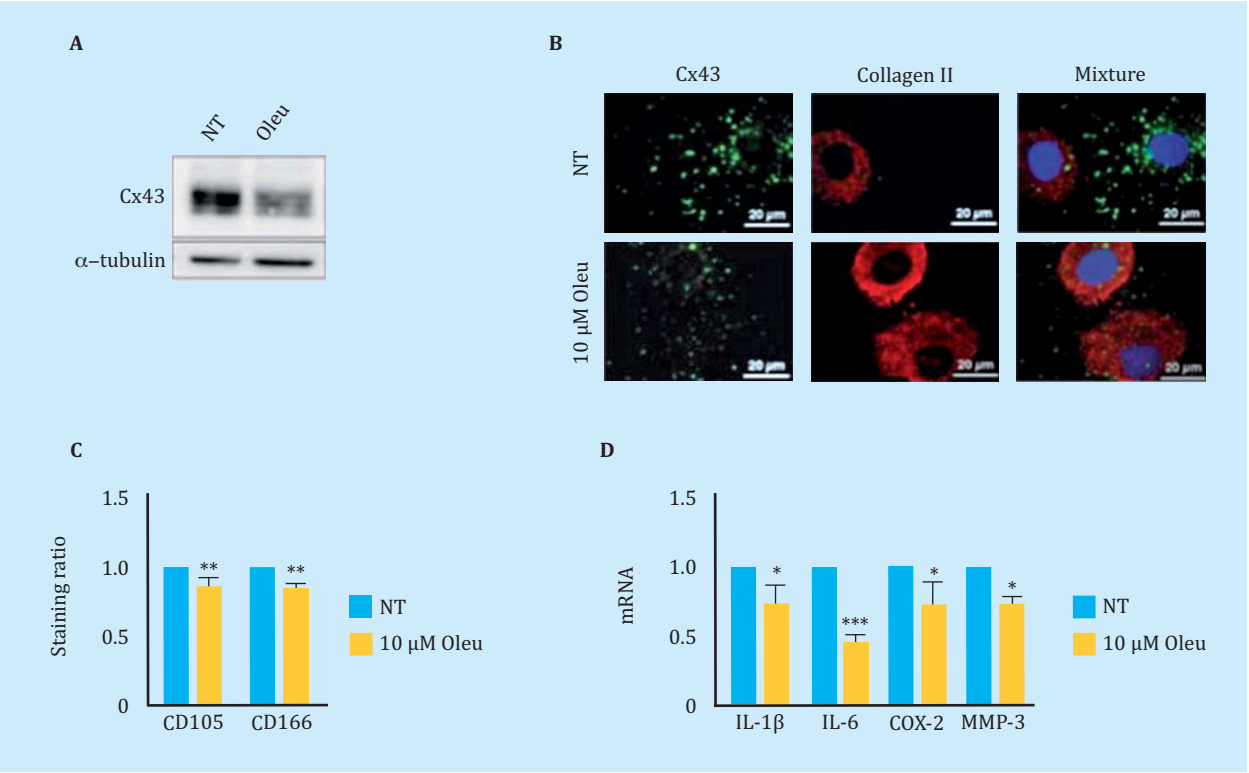


Figure 4. Sections of three-dimensional culture of arthritic chondrocytes (OAc) cultured in chondrogenic medium (MC) with/without 10 µM oleuropein for 30 days. In the upper panel, immunohistochemistry of a micromass for type II collagen (n=4-6, mean ± SEM, *p<0.05, Student's t-test). Below, staining of toluidine blue to detect proteoglycans, which produce a blue to pink-violet color shift (n=6, mean ± SEM, **p<0.01, Mann-Whitney test)

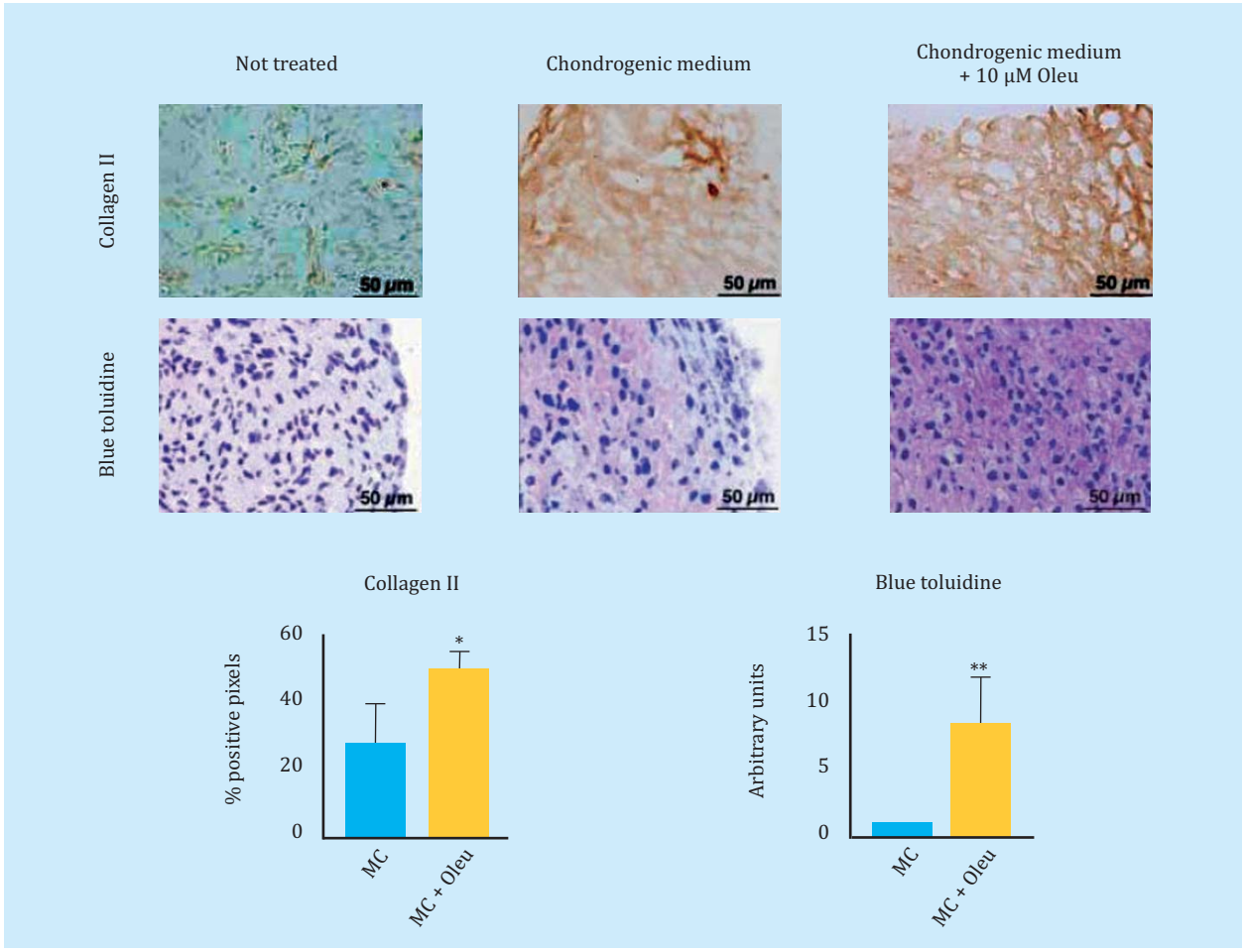


Figure 5. (A) Western blot comparing levels of PCNA, NF-Kβ, and nuclear Twist-1 in chondrocytes that over-express Cx43 (T/C-Cx43) with respect to the same chondrocytes transfected with a control plasmid (T/C). (B) Western blot comparing total p53 levels between chondrocytes overexpressing Cx43 (T/C-Cx43) and control chondrocytes (T/C). (C) Gene expression of p16 of chondrocytes overexpressing Cx43 with respect to control cells (n=4, mean ± SEM, *p<0.05, Mann-Whitney test). (D) Above, β-galactosidase staining associated with senescence measured by X-gal rupture in arthritic chondrocytes (OAc) treated with 10 µM oleuropein for 7 days. Below, quantification by flow cytometry of β-galactosidase levels after the same treatment (n=5, mean ± SEM, *p<0.05, Mann-Whitney test)

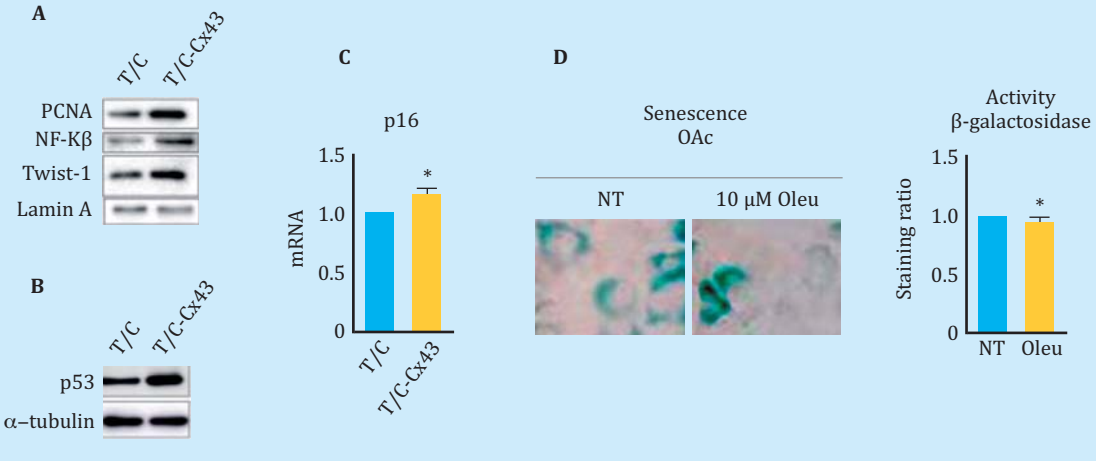
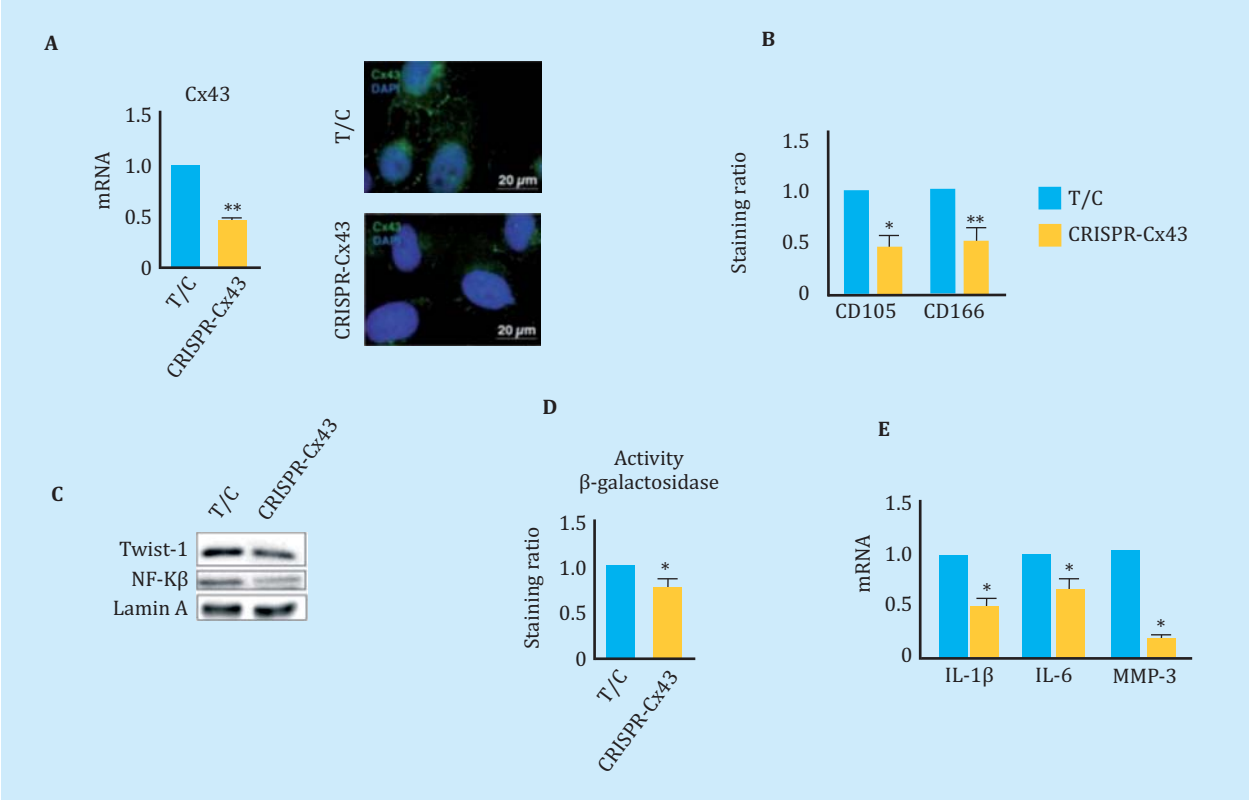


Figure 6. (A) On the left, gene expression levels of Cx43 in chondrocytes T/C-28a2 (T/C) and the same line with only one copy of Cx43 (CRISPR-Cx43). n=4, mean ± SEM; *p<0.05; Mann-Whitney test. On the right, immunofluorescence for Cx43 (green) in chondrocytes T/C-28a2 (T/C) and the same line transfected with only one copy of Cx43 (CRISPR-Cx43). The nuclei have been stained with DAPI (blue). (B) Levels of the CD105 and CD166 markers measured by flow cytometry in the T/C-28a2 cell line with a single copy of Cx43 (CRISPR-Cx43) compared to the same line without transfection (T/C). n=7, mean ± SEM; *p<0.05, **p<0.01; Mann-Whitney test (C) Western blot to detect Twist-1, NF-kβ and N-Cadherin comparing a nuclear extract of the T/C-28a2 (T/C) line and from the same line with low amount of Cx43 (CRISPR-Cx43). Lamina protein A has been used as a load control. (D) Quantification by flow cytometry of β-galactosidase levels in the T/C-28a2 line (T/C) and the same line with only one copy of Cx43 (CRISPR-Cx43). n=4, mean ± SEM; *p<0.05; Mann-Whitney test. (E) Gene expression levels of IL-1β, IL-6 and MMP-3 measured in the same cells. n=4, mean ± SEM; *p<0.05; Mann-Whitney test)



MEC in *in vitro* tests. The decrease in Cx43 gave rise to cellular re-differentiation and, therefore, to a lower expression of pro-inflammatory cytokines and degrading enzymes of the articular cartilage matrix. Our results also show that high levels of Cx43 in chondrocytes are related to an increase in senescence associated with a higher expression of $p16^{INK4a}$ and high levels of p53. Recent studies highlight the importance of senescence in osteoarthritis[29-33]. In fact, Jeon et al. published an article in Nature Medicine where they showed senescence as a new therapeutic target to treat osteoarthritis and promote the regeneration of cartilage[33,34]. In this study we demonstrate for the first time the relationship between the over activity of Cx43 in human chondrocytes and the activation of dedifferentiation and cellular senescence that lead to alterations in the regeneration process and favor the progress of the disease. From these results, therapies aimed at decreasing Cx43 levels in osteoarthritis arise as an interesting therapeutic approach for osteoarthritis.

In conclusion, these findings suggest that the increase in Cx43 activity reached from very early stages of OA[6] could contribute to the degeneration of articular cartilage and joint by activating cellular dedifferentiation via TEM and cellular senescence, contributing to the synthesis of enzymes that degrade the release of cytokines that contribute to the degenerative process in the joint. These results demonstrate that Cx43 and UCs act as a regulator of dedifferentiation/re-differentiation and senescence in chondrocytes, probably activating proteins related to TEM, such as Twist-1, and pro-inflammatory cytokines such as IL-1β. The decrease in Cx43 levels in OAc promotes its re-differentiation, decreasing the expression of inflammatory mediators and senescence, and in turn is accompanied by a greater deposition of Col2A1 and proteoglycans in the extracellular matrix. The use of molecules such as oleuropein and the design of studies to decrease the activity of Cx43 *in vivo* is probably a first step in the development of innovative therapeutic strategies for the effective treatment of osteoarthritis from early stages of the disease by restoring tissue regeneration.

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Qualitative and quantitative status of general bone in osteonecrosis of the jaws. Effect of bisphosphonates

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Summary

Objetive: Osteonecrosis of the jaw (ONJ) is a recently reported disease whose origin and development are unknown, although prolonged bisphosphonate treatment has been attributed, among other causes. While ONJ is a localized condition, the action of bisphosphonates is widespread and affects all bones. No studies show the general bone status of patients with ONJ. Our study examines the general condition in patients with ONJ using quantitative measurements and qualitative estimates of bone by means of bone mineral density (BMD) and trabecular bone score (TBS) and ultrasound parameters in the calcaneus (QUS), along with other diseases and the taking of drugs (especially bisphosphonates) in patients with ONJ who may be involved in the pathogenesis.

Material and method: Observational and cross-sectional study of cases and controls, conducted in 304 patients of both sexes, in which the case group (group I) was formed by 24 patients who had suffered ONJ. The control group (group II) contained 280 patients who did not present ONJ and who received bisphosphonates over at least 5 years for various reasons. All of them underwent bone densitometry (DXA, Hologic 4500 Discovery®) in the lumbar spine and proximal femur. In addition, TBS measurements were made in the lumbar spine, as well as ultrasound parameters in the calcaneus (Hologic, Sahara®) in the dominant foot (QUS).

Results: Patients suffering ONJ presented greater comorbidity than controls, with a higher prevalence of diabetes mellitus, cancer, rheumatoid arthritis, hyperthyroidism, heart disease, arrhythmias, heart failure and hypercholesterolemia. Therefore, the consumption of corticosteroids, (oral and inhaled), anticoagulants, hypnotics, bisphosphonates i.v. (zoledronate), and antineoplastic chemotherapy was also higher among patients with ONJ than control patients. However, among the patients with ONJ the percentage taking oral bisphosphonates was lower. Densitometric values (BMD measured in lumbar spine L2-L4, femoral neck and total hip) were higher in patients with ONJ compared to those in controls. The TBS showed no statistically significant differences between the two groups, and the ultrasound showed higher values of QUI and SOS in patients with ONJ than in controls. The prevalence of fragility fractures was similar in both groups.

Conclusions: Patients with ONJ in our study presented greater comorbidity and a higher consumption of drugs than the patients in the control group, except for oral bisphosphonates. On the other hand, both BMD and ultrasound showed higher values in patients with ONJ than in controls. If we consider DXA as a technique for measuring the amount of bone mass, and TBS and calcaneal ultrasound estimating qualitative aspects of bone, we could assume that neither bone quantity nor quality in general seems to be affected in ONJ, and that its etiopathogenic mechanism is probably another. Oral bisphosphonates do not appear to be among the drugs involved in ONJ's origin and development, but the most potent and intravenously administered bisphosphonates are, although they cannot be considered independently of the underlying disease for which they are administered.

Key words: osteonecrosis, jaws, bisphosphonates, quality, quantity, densitometry, ultrasound.

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Introduction

Osteonecrosis of the jaw (ONJ) is a disease described fairly recently. After the reported findings by Marx[1], bisphosphonates were considered the etiological agent responsible for the disease, even being called osteonecrosis due to bisphosphonates[2-5], which is wrong since many factors in addition to these drugs may be implicated in the etiopathogenesis of ONJ[1,6,7].

One of the hypothesis about ONJ's development would be the existence of an excess suppression of bone remodeling, which can be produced by bisphosphonates or by other potent anti-resorptives, such as denosumab, a drug that is also involved in ONJ[8,9]. Since these drugs act on the entire skeleton, if there is such an excess of oversupression of bone remodelling, one could expect the existence of alterations in both the amount of BMD and bone quality in other locations. Although there are many descriptions of isolated cases or series of this disease in the literature, outlining its clinical characteristics and possible association with different diseases and risk factors[1,3-7,10], we have not found publications that analyze the possible quantitative alterations and/or qualitative bone in patients with ONJ.

Bone mass measurement by dual radiological absorptiometry (DXA) has been sufficiently validated and is accepted as a reliable bone quantification technique by measuring bone mineral density (BMD)[11-14]. However, non-invasive bone quality measurement techniques have not been as successful, due to the many aspects that the concept of bone quality encompasses. Despite this, there are currently two techniques that can estimate some aspects of bone quality. On the one hand, trabecular bone score (TBS), associated with DXA, which offers information on bone microstructure[15-21]; and on the other, quantitative ultrasound (QUS), which although it is not known exactly what bone properties it reflects, its measurements have also been related to bone microarchitecture and some mechanical parameters[22-24].

Thus, our research objective has been to study the possible alterations in the amount of bone tissue in locations other than the jaws and that serve as a reference, measured as BMD by bone densitometry (dual radiological absorptiometry, DXA) in the lumbar spine and proximal femur; as well as in bone quality, estimated, on the one hand, by means of TBS and, on the other, using the parameters obtained by QUS, in a population of patients suffering from ONJ and estimating the presence of certain diseases and treatment that affect the bone. We highlight bisphosphonates as a secondary objective, which could participate in its etiopathogenesis. For this, we take as a reference a control group of patients who had received bisphosphonates for at least 5 consecutive years and who continued to take them at the time of the study.

Material and method

Inclusion criteria

We have carried out a case and controls study in which we consider "case" to patients who had suffered an ONJ and "controls" to patients without ONJ and who had received bisphosphonates for a minimum of 5 years and continued taking it today. .

We include as cases 24 patients who were diagnosed with ONJ following the criteria of the "International Task Force on Osteonecrosis of the Jaw"[25]. The controls were subjects without ONJ who were recruited among patients studied in the Bone Metabolic Unit of the Insular University Hospital and who had received oral or intravenous bisphosphonates (i.v.) for a minimum of 5 years and continued receiving them.

Physical examination

All participating patients underwent a complete physical examination. Their height was obtained on a height rod and weight on a scale, with the patient wearing light clothes, without shoes. The body mass index (BMI) was calculated from the formula = weight (kg)/height (m)^2.

Dual radiological absorptiometry or bone densitometry (DXA)

BMD was estimated using a Hologic® QDR 4500 Discovery densitometer (Hologic, Spain). The determinations were made in the lumbar spine (L2-L4 vertebrae) and in the proximal femur (femoral neck, trochanter, intertrochanter and total femur). The computer program provided by the manufacturer allows us to separate the anatomical locations. The results were expressed in g/cm^2 and T-score. The accuracy of the equipment (coefficient of variation) was 0.5% *in vitro* (measured with a standard phantom) and 0.9% *in vivo* (obtained by double measurements made in 12 patients on the same day). All determinations were made by the same operator, so there were no inter-observer variations. T-score values were calculated from the reference values that the device includes obtained for the Spanish population.

Trabecular bone score (TBS)

All TBS measurements were carried out using the TBS iNsight Software program, version 2.0.0.1 (Med-Imaps, Pessac, France). The computer program uses the image previously obtained by DXA in the same region of interest (lumbar spine, L2-L4). T-score values were calculated from the reference values obtained for the Spanish population[26].

Quantitative ultrasound (QUS)

All patients underwent an ultrasound on the calcaneus of the dominant foot. For this, we use the Sahara® Clinical Sonometer ultrasound device (Hologic Inc., Bedford, Massachusetts. USA). The system consists of 2 transducers, one of which acts as an emitter and the other as an ultrasound receiver. The parameters obtained are ultrasonic broadband attenuation (BUA) and sound speed (SOS). The results obtained by both parameters, BUA and SOS are combined to obtain the so-called ultrasonic quantitative index or QUI, when applying the formula:

QUI = 0.41 X (BUA + SOS) - 571.

In all ultrasound determinations, their corresponding T-scores were calculated with the data obtained as reference values for the Spanish population[27].

Diagnosis of fractures

All patients underwent an AP and lateral dorsal and lumbar spine Rx. The prevalent vertebral fractures were diagnosed by applying the semi-quantitative Genant classification of vertebral fracture[28]. The presence of non-vertebral fractures was documented from the clinical history obtained from the patients confirmed by hospital medical records or by means of the appropriate radiographic studies.

Statistic analysis

Continuous data were expressed as means and standard deviations when the variables followed a normal distribution, or through the medians with their interquartile ranges when the distribution was not normal. Categorical variables

were expressed as frequencies and percentages. For independent data, the percentages were compared using the Chi-Square test (χ^2) or the exact Fischer test. The averages were compared using Student's t test and the medians applying Mann Whitney's U. In all cases the level of statistical significance was considered at 5% (p value<0.05).

Ethical and legal aspects

The project was approved by the Ethics and Clinical Trials Committee of the Insular University Hospital, Gran Canaria, Spain. This is an observational study in which there was no pharmacological intervention of any kind. We observed the recommendations of the World Medical Association contained in the Declaration of Helsinki[29] throughout the study.

Results

Table 1 shows the baseline characteristics and lifestyles of the patients included in the study. The patients were similar in age and the proportion of men and women was similar in both groups.

The patients with ONJ was shorter in height, presented a higher BMI and a lower consumption of tobacco and alcohol than controls. We do not observe statistically significant differences in coffee consumption or physical activity in leisure time.

Table 2 shows the comorbidity of patients with ONJ and controls. Patients who suffered ONJ had a greater comorbidity than controls: they showed a higher prevalence of diabetes mellitus, cancer, rheumatoid arthritis, hyperthyroidism, heart disease, arrhythmias, heart failure and hypercholesterolemia. The prevalence of fragility fractures was similar in both groups.

In table 3, we show the consumption of drugs of both patient groups. In line with the existence of greater comorbidity, patients with ONJ had a significantly higher consumption of oral cortico-steroids, oral anticoagulants and hypnotics than controls and, similarly, a greater number of them had received chemotherapy. Inhaled steroid consumption also showed a trend that was very close to reaching the level of significance (p=0.05).

Table 1. Baseline characteristics and lifestyles of patients with ONJ and controls

	Cases ONJ (n=24)	Controls (n=280)	p value
Age (years)	69.3 ± 11.4	69.1 ± 10.4	0.900
Sex			0.325
Men	4 (16.7%)	30 (10.7%)	
Women	20 (83.3%)	250 (89.3%)	
Weight (kg)	65.9 ± 16.4	68.8 ± 16.1	0.425
Size (cm)	152 ± 11.3	157 ± 9.3	0.010
Wingspan (cm)	159.4 ± 15.9	160 ± 10.9	0.853
BMI (kg/m^2)	28.8 ± 7.3	25.9 ± 6.6	0.019
Current calcium intake (mg/day)	725 (390.5)	700 (250)	0.459
Tobacco			0.049
Yes	1 (4.2%)	49 (17.6%)	
No	16 (66.7%)	192 (68.8%)	
Former smoker	7 (29.2%)	38 (13.6%)	
Alcohol			0.022
Yes	4 (16.7%)	94 (33.7%)	
No	18 (75%)	181 (64.9%)	
Former drinker	2 (8.3%)	4 (1.4%)	
Coffee			0.777
Yes	21 (87.5%)	231 (83.1%)	
No	3 (12.5%)	47 (16.9%)	
Physical activity at leisure			0.902
Low	16 (66.7%)	173 (62%)	
Half	7 (29.2%)	92 (33%)	
Intense	1 (4.2%)	14 (5%)	

Continuous variables are summarized as mean ± standard deviation or as medians with their interquartile intervals (IQR). Categorical variables are expressed as frequency (%). BMI: body mass index: = (weight/size^2).

Table 2. Comorbidity of patients with ONJ and controls

	ONJ (n=24)	Controls (n=280)	p value
Mellitus diabetes			
Yes	5 (23.8%)	21 (7.5%)	0.025
No	16 (76.2%)	259 (92.5%)	
Cancer			
Yes	11 (45.8%)	31 (11.1%)	<0.001
No	13 (54.2%)	249 (88.9%)	
Rheumatoid arthritis			
Yes	9 (37.5%)	7 (2.5%)	<0.001
No	15 (62.5%)	273 (97.5%)	
Thyroid disease			
Hyperthyroidism	3 (12.5%)	7 (2.5%)	0.027
Hypothyroidism	1 (4.2%)	24 (8.7%)	
Any	20 (83.3%)	246 (88.8%)	
Heart disease			
Yes	11 (45.8%)	33 (11.8%)	<0.001
No	13 (54.2%)	247 (88.2%)	
Angina			
Yes	1 (4.2%)	14 (5%)	1
No	23 (95.8%)	266 (95%)	
Myocardial infarction			
Yes	1 (4.2%)	4 (1.4%)	0.339
No	23 (95.8%)	276 (98.6%)	
Arrhythmias			
Yes	6 (25%)	17 (6.1%)	0.005
No	18 (75%)	263 (93.9%)	
Heart failure			
Yes	5 (20.8%)	8 (2.9%)	0.002
No	19 (79.2%)	272 (97.1%)	
Fragility fractures			
Yes	11 (45.8%)	104 (37.1%)	0.511
No	13 (54.2%)	176 (62.9%)	
Hypercholesterolemia			
Yes	133 (47.5%)	3 (12.5%)	0.001
No	147 (52.5%)	21 (87.5%)	

The results are expressed as frequencies (%).

In patients with ONJ, the use of bisphosphonates was mostly via i.v. (75%) and much less orally (8.3%). Furthermore, 16.7% of these patients had never taken or received bisphosphonates. Obviously, since it was an inclusion criterion, in the control group 100% had received or taken bisphosphonates, being mostly oral (92.1%).

In table 4, we present the densitometric, ultrasound parameters and TBS values. BMD showed higher values in patients with ONJ in all anatomical locations where it was determined; both in the lumbar spine and in the proximal limb of the femur, the T-score was also higher. We did not obtain statistically significant differences in the values of TBS and Broadband Ultrasound Attenuation (BUA), while patients with ONJ showed higher values of QUI and SOS than controls.

According to the densitometric values ((DXA) observed at the time of the study, we obtained that 28% of patients with ONJ had osteoporosis criteria (T-score ≤-2.5 in any of the following locations: L2-L4 , femoral neck or

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Table 3. Drugs used by patients with ONJ and controls

	ONJ (n=24)	Controls (n= 279)	p value
Oral corticosteroids			0.027
Nowadays	5 (20.8%)	17 (6.1%)	
Sometime (>6 months)	2 (8.3%)	22 (7.9%)	
Never	17 (70.8%)	240 (86.0%)	
Inhaled steroids			0.050
Nowadays	3 (12.5%)	8 (2.9%)	
Sometime (>6 months)	1 (4.2%)	9 (3.2%)	
Never	20 (83.3%)	262 (93.9%)	
Chemotherapy			<0.001
Yes	11 (45.8%)	14 (5%)	
No	13 (54.2%)	266 (95%)	
Statins			0.255
Nowadays	4 (16.7%)	92 (33.0%)	
Sometime (>6 months)	2 (8.3%)	17 (6.1%)	
Never	18 (75.0%)	170 (60.9%)	
Oral anticoagulants			0.003
Nowadays	4 (16.7%)	8 (2.9%)	
Sometime (>6 months)	0 (0.0%)	7 (2.5%)	
Never	20 (83.3%)	264 (94.6)	
Hypnotics			0.006
Nowadays	18 (75.0%)	115 (41.2%)	
Sometime (>6 months)	2 (8.3%)	70 (25.1%)	
Never	4 (16.7%)	94 (33.7%)	
Bisphosphonates			0.001
Oral	2 (8,3%)	259 (92.1%)	
i.v. zoledronate	18 (75%)	22 (7.9%)	
Never	4 (16,7%)	0 (0%)	

The results are expressed as frequencies (%).

total hip), while these criteria were appreciated in 47.6% of the control patients, without the difference being significant (p=0.06) (Table 5).

Discussion

ONJ is a relatively recent disease, the first series having been described about 15 years ago[1,30-32]. Its causal mechanism is unknown[3,6,7,32-35] and many possible risk factors have been related, but without establishing an unequivocal cause-effect with any of them[2,3,5-7,36,37], which can be considered multifactorial. For a long time, treatment with bisphosphonates has been pointed out as a primary etiologic agent of ONJ, to the point that for some time ONJ was called bisphosphonate-induced osteonecrosis[1-4,31,32,35,38]. This is still considered by many dental specialists who indicate their withdrawal before orodental surgery.

An etiopathogenic hypothesis that was accepted for a long time indicated that bisphosphonates, administered over several years and at high doses, would produce an excessive suppression of remodeling, which would imply bone development with a smaller amount of bone mass and a severe alteration of quality, what came to termed "frozen" bone[39]. In support of this hypothesis, it has been observed that the vast majority of patients with ONJ, more than 95%, are patients who have suffered from cancer and who in addition to the basic treatment of the process (surgery, radiotherapy) have received polychemotherapy and high-dose intravenous bisphosphonates, usually zoledronate[1,6,7,30] at the oncological dose, which is 4 mg i.v. every 28 days (52 mg per year), while in the treatment of osteoporosis, the dose used of the same drug is 5 mg i.v. once a year[40].

However, there are also some disagreements. First, a considerable percentage of patients with ONJ, 16.7%, had never received bisphosphonates.

On the other hand, the greater BMD measured by DXA in all the locations of these patients with respect to the control patients points to a greater general bone quantity of the former versus the latter. We have not found in

the main databases similar studies to ours comparing BMD in patients with ONJ with controls in treatment with bisphosphonates. So we do not know whether or not this finding has been corroborated by other authors. We want to highlight the fact that there was no statistically significant difference in the densitometric diagnosis of osteoporosis between both groups. It may seem logical that there should be a higher percentage of osteoporosis diagnosis among the control group, since treatment with oral bisphosphonates (drug of choice for osteoporosis) was the majority. However, we must bear in mind that long-term treatment with this drug increases BMD, and therefore the T-score, causing its values to depart from the densitometric criteria of osteoporosis.

We also wanted to consider bone quality, a much more controversial aspect, since there is no single definitive and non-invasive method that has been accepted as the "gold standard" for estimating bone quality, unlike what happens with densitometry, which is the universally accepted reference for quantity[11-14]. One of the recently described methods for estimating bone quality is the so-called trabecular bone score or TBS[16], which basically evaluates the integrity of the vertebral bone trabeculae, reanalyzing the DXA images[17-21]. The parameters obtained with QUS have also been proposed as possible indicators of bone quality[22-24]. In our series, patients with ONJ showed similar values of TBS and BUA to those of control patients, and the SOS and QUI rates were slightly but significantly higher in the former. This leads us to believe the qualitative bone aspects in both groups were similar, and, if anything, never worse in patients with ONJ than in those taking bisphosphonates. Some authors have described low values of ultrasound parameters in patients with ONJ[41], but as with densitometry in ONJ, there are very few studies similar to ours with which to make comparisons. If we take into account that the control patients took bisphosphonates for a long time and considering that bisphosphonates improve bone quantity and quality[42,43], the higher values of BMD, TBS, SOS and QUI in patients with ONJ could indicate that the general bone health of these patients is adequate.

Finally, if we add to the previous results the fact that the prevalence of fragility fractures was also similar in both groups (and considering that bisphosphonates decrease the risk of fracture), we have indirect evidence that the general bone structure, both quantitatively and qualitatively, it is the less similar (if not better) in patients with ONJ and patients under treatment with bisphosphonates.

We totally agree that the cause and development of ONJ is multifactorial, as has been published in multiple studies and agreed by consensus[1-3,7,10,36]. As we have observed in

Table 4. Bone parameters related to qualitative and quantitative aspects: BMD measured in the lumbar spine and proximal limb of the femur, TBS measured in the lumbar spine and ultrasound parameters obtained in the calcaneus

DXA	ONJ (n=24)	Controls (n=280)	p value
L2-L4 (g/cm^2)	0.95 (0.18)	0.82 (0.18)	0.001
T-score L2L4	-0.87 (1.57)	-2.22 (1.76)	0.001
Femoral neck (g/cm^2)	0.68 (0.23)	0.64 (0.15)	0.025
T-score femoral neck	-1.46 (2.15)	-1.89 (1.36)	0.006
Total femur (g/cm^2)	0.86 (0.24)	0.78 (0.17)	0.006
T-score total femur	-0.45 (-0.64)	-0.5 (1.33)	0.006
TBS L2L4 (g/cm^2)	1.18 (0.14)	1.25 (0.13)	0.174
T-score TBS L2L4	-3.32 (1.72)	-2.47 (1.69)	0.174
QUI	72.4 (18.7)	69.9 (26.4)	0.040
T-score QUI	-1.68 (0.52)	-1.83 (1.34)	0.040
BUA (dB/MHz)	60.8 (24.2)	57 (23.5)	0.119
T-score BUA	-1.07 (0.12)	-1.32 (1.56)	0.119
SOS (m/s)	1511.8 (33.5)	1508 (39.6)	0.033
T-score SOS	-1.96 (0.72)	-1.94 (1.2)	0.033

The results are expressed as medians and interquartile intervals (IQR).

Table 5. Percentage of densitometric diagnosis of osteoporosis at the time of the study in both groups

	ONJ group	Group control	P value (Chi Squared)	OR (IC 95%)
Densitometric osteoporosis	7 (28%)	130 (47.6%)	0.060	2.338; (0.946, 6.777)
No densitometric osteoporosis	18 (72%)	143 (52.4%)		

our series, patients with this disease have greater comorbidity. Therefore, drug use is also significantly higher in this group of patients[44] than among those studied here: oral corticosteroids, inhaled corticosteroids, oral anticoagulants, hypnotics and having received polychemotherapy. However, if we focus on bisphosphonates, patients with ONJ have a greater use of potent bisphosphonates via the i.v. (75%), which was closely related to the higher incidence of cancer, but not oral bisphosphonates.

One of the limitations of this study is the sample size of the cases, with only 24 patients. This is due to the low incidence of this disease and the difficulty of getting participation in a study of these characteristics of some patients, due to its delicate clinical situation. On the other hand, the choice of the control group could be discussed. We have chosen patients who had been receiving bisphosphonates for at least 5 years, given that it is precisely this fact that is considered a risk factor associated with the appearance of ONJ[45-47], but which have a beneficial effect on bone in general. Finally, we are aware that unvalued local circumstances, such as oral hygiene, or the presence of dentogingival diseases or dental interventions, have a relevant and decisive specific weight in ONJ pathogenesis, although we do not include them because they are localized circumstances that do not affect the bone in general.

Conclusion

Our study results indicate that patients who have suffered ONJ do not appear to have worse bone health (in terms of quantity and quality) in general compared to patients who have been receiving bisphosphonates continuously for at least 5 years. In addition, oral bisphosphonates were not the most used drugs among these patients, so we have to pay more attention to more prevalent ones such as corticosteroids, intravenous bisphosphonates, chemotherapy, hypnotics and oral anticoagulants; while taking into account comorbidities, such as cancer, diabetes, rheumatoid arthritis, hypercholesterolemia, heart disease and thyroid conditions.

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Osteogenic cells affected by soluble tumor factors contribute to bone pre-metastatic niche formation

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Summary

Objective: To analyze the effect of the secrets of solid organotropic tumors towards bone in osteogenic, osteoblastic and osteocytic lineage cells, in the expression of genes related to bone metabolism.
Material and method: We characterize the changes in gene expression by quantitative real-time PCR of the OPG/RANKL axis, as well as other genes related to osteoblastic differentiation such as Runx2 and osteocalcin, induced by the conditioned means of prostate tumor cells, breast and melanoma in pre MC3T3-E1 osteoblasts and murine MLO-Y4 osteocytes or in human osteoblasts, as appropriate by species.
Results: Stimulation of osteocitic cells with conditioned means of melanoma or prostate adenocarcinoma cells induced an increase in OPG and RANKL gene expression, with the OPG/RANKL ratio being increased. Only the secretome of prostate adenocarcinoma cells altered the expression of Runx2 in osteocytes. Conditioned media of breast cancer cells only modified the expression of RANKL in osteoblast cells, with a decrease in OPG/RANKL ratio.
Conclusion: Soluble tumor factors have osteocitic cells as their cellular target, favoring the induction of a pre-metastatic bone niche by modifying the OPG/RANKL ratio in the bone environment, and, thus, the progression of bone organotropic tumors such as melanoma and prostatic adenocarcinomas.

Key words: bone organotropic tumors, soluble tumor factors, pre-metastatic bone niche, bone metastases, osteocytes and osteoblasts.

Introduction

The appearance of metastatic disease seriously threatens the survival rate of patients who develop a tumor. Certain types of tumors have been found to present a high tendency to colonize specific organs. From the hypothesis formulated by Paget ("seed-and-soil")[1], few studies have deciphered the regulatory mechanisms of metastatic organotropism. Initial studies focused on the function of the intrinsic properties of the tumor cell, such as gene expression and colonization regulation pathways, in the direction of organotropism[2-4].

Bone is an organ frequently infiltrated by the metastatic spread of solid tumors[5,6]. The appearance of metastatic disease is a serious threat in the survival rate of patients who develop a tumor. From 65-80% of subjects with prostate cancer or metastatic breast present skeletal complications[5]. The study of bone metastases has mainly focused on the interaction of the tumor cell with the bone, once the metastasis has been established, ignoring the subclinical stages of the process that occurs previously. The establishment of tumor cells in the bone microenvironment alters the balance of the bone remodeling process between bone formation, induced by osteoblasts, and osteoclast-mediated resorption. Consequently, the survival and proliferation pathways of tumor cells are favored, inducing the formation of "a vicious cycle of bone metastases"[7].

Though not exclusive, tumors cause two different types of skeletal lesions. The most common form, represented by breast cancer is the osteolytic lesion associated with an alteration of bone remodeling with an increase in osteoclastic activity and subsequent osteolysis[8-11].

A second type of so-called osteoblastic lesions is characterized by high bone remodeling with an increase in osteoblast activity with an increase in osteoid and mineralization rate. These areas of de novo-formed bone in areas of metastasis are called osteoesclerotic lesions, which are usually weak and unstable, with a tendency to break. This type of lesions is characteristic of prostate cancer. However, the existence of a osteoclast mediated component is currently recognized as a previous step for the establishment of osteoblastic lesions[12,13].

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Recent studies have described pro-metastatic changes in organs where metastases will later appear. Such changes induce the formation of pre-metastatic niches that favor the implantation of tumor cells in target organs[14,15].

The cellular complexity of the bone (osteocytes, osteoblasts, osteoclasts, bone covering cells, endothelial cells and hematopoietic tissue), as well as the functions they carry out in regulating the metabolism and bone remodeling, raises the possibility that the formation of the pre niche-metastatic bone is the result of a complex network of combined and sequential modifications and alterations of all these cells[16].

Despite the existence of some observations analyzing the effect of the factors secreted by tumor cells that affect the viability of bone cells[17], the existence of common mechanisms or changes in bone cells induced by solid tumors with high organotropism is unknown. He made the bone as a metastatic target organ.

In this study, we have analyzed the changes in the transcriptional profile of cytokines related to bone metabolism in osteoblastic and osteocitic cells, induced by soluble tumor factors of breast, prostate and melanoma tumor cells. Our observations show that these factors significantly modify the transcriptional profile of osteocytes. These results suggest a relevant role of osteocytes as the initial inducing cell in the formation of the pre-metastatic bone niche.

Material and method

Cell cultures

The MC3T3-E1 murine pre-osteoblastic cell lines (ATCC: CRL-2593) and MLO-Y4 murine osteocitic cells (donated by Lynda Bonewald) were cultured in DMEM with 10% bovine fetal serum (SFB) or α-MEM with 2.5% fetal sheep serum (SCF) and 2.5% SFB, respectively. All cells were cultured in media containing penicillin (100 units/mL) and streptomycin (100 µg/mL) in the humidified incubator at 37°C and 5% atmospheric CO_2.

The hFOB 1.19 human osteoblastic continuous line (ATCC® CRL-11372TM) was grown in a 1:1 mixture of Ham's F12 and DMEM with 2.5 mM L-glutamine, 0.3 mg/mL G418 and 10% SFB in the humidified incubator at 34°C and 5% atmospheric CO_2.

We use the TRAMPC-1 mouse prostate adenocarcinoma (ATCC® CRL-2730TM), murine melanoma B16 (ATCC® CRL-6323) and human breast cancer (ATCC® MDA-MB-231 HTB-26) lines to obtain of tumor secretoma. These cells were cultured in DMEM culture medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 1% glutamine and 10% SFB. When the culture reached confluence, they were washed with a phosphate solution (PBS). Next, tumor secrets and bone conditioned media used for stimulation of previously activated tumor cells with soluble bone factors were obtained..

To obtain the conditioned media of both tumor cells and bone cells, the cell lines were grown to confluence with α-MEM culture medium supplemented with penicillin and 0% streptomycin of SFB, after 24 hours of incubation, the media were collected and cell debris and dead cells were removed by centrifugation (5,000 rpm, 10 minutes).

To avoid any type of cross-biological reactivity of cytokines of one species in cells of another species[18] and reproduce as faithfully as possible the communication of tumor cells with bone cells, our *in vitro* experimental models were designed according mainly to the criteria of species. In this way, the effects of the tumor secretoma of the human breast cancer line on the human osteoblastic line were evaluated. There is currently no well characterized osteocitic continuous line. The effects of the secrets of prostate and mouse melanoma tumor lines were studied on bone murine lines (osteoblastic, MC3T3, and osteocitic, MLOY4). Figure 1 shows the protocol used to obtain conditioned media and its stimulation in the different 25% cell lines.

Cell viability assay

The number of viable bone cells MC3T3, MLOY4 and hFOBs stimulated or not with 25% tumor secretoma was evaluated by the trypan blue exclusion test as previously described[19].

Gene expression studies by real-time PCR

Total RNA was extracted from cell cultures by the Trizol method. CDNA synthesis was performed using the reverse transcriptase of the avian myeloblastosis virus (Promega) and random hexamer primers. Real-time PCR was carried out in the ABI PRISM 7500 system (Applied Biosystems) using Sybr premix ex Taq (Takara, Otsu, Japan) and specific primers of each gene (Table 1). All results were expressed in number of mRNA copies calculated for each sample using the cycle threshold value (Ct). The relative gene expression is represented as: 2-ΔΔCt, where ΔΔCt = ΔCtgen target - ΔCt18S/GAPDH. The change in the number of times with respect to treatment is defined as the expression compared to the control, calculated as 2-ΔΔCt, where ΔΔCt = ΔCreating - ΔCcontrol. The specificity of the amplicon was confirmed as the presence of a single amplification after melting curve analysis. The results shown correspond to the average of at least 3 independent experiments in triplicate.

Statistical analysis

Data were expressed as mean ± standard error. The differences between experimental conditions and controls were analyzed using the Mann Whitney U test where the values of $p<0.05$ were considered significant.

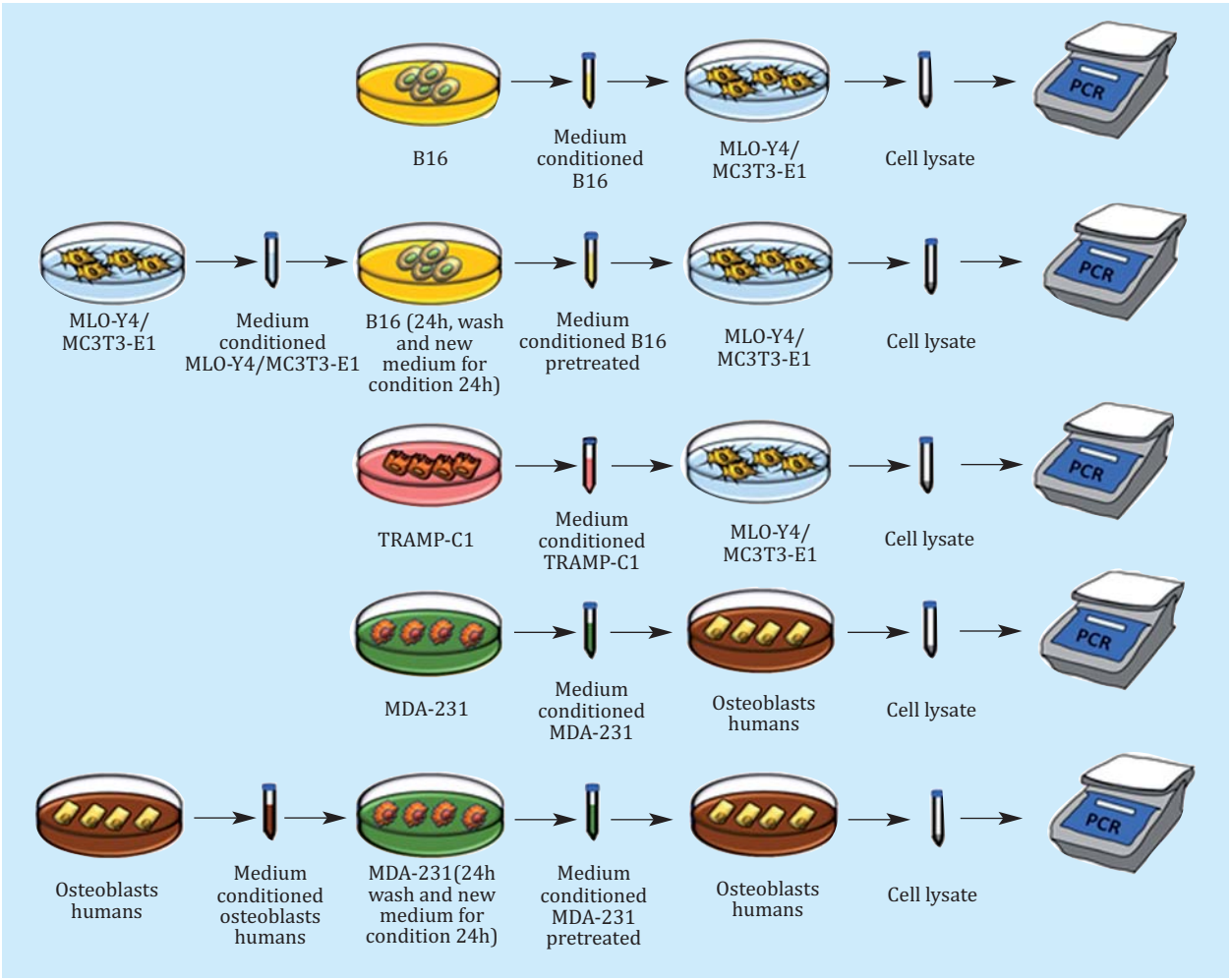
Results

Communication by soluble factors between tumorigenic melanocytes and bone cells regulates the expression of bone remodeling genes in osteocytes

In order to study the communication between tumor cells and bone cells in the formation of pre-metastatic bone niches, mouse or human bone cells were stimulated with the secrets of different tumor cells with organotropism to bone tissue, which had previously been stimulated or not with osteomas or osteoblast secrets (Figure 1).

First, we corroborate that the conditioned tumor media of the TRAMPC-1 or B16, that is to say tumor secretoma, does not affect the viability of the osteoblast cells, MC3T3, and osteocitic, MLOY4 after 24 h of stimulation (Figure 2). However, stimulation of MLO-Y4 osteocytes with secrets obtained from conditioned media of B16 melanoma cells induced overexpression of the OPG gene with a net increase in the OPG/RANK-L ratio, without significantly affecting the gene expression of RANK-L, nor to the transcription factor Runx2 (Figure 3A-D). In contrast, the secrets of B16 melanoma cells, which had previously

Figure 1. Graphical representation of the work protocol used to obtain pre-treated or not conditioned means (MC). Untreated MLO-Y4, MC3T3-E1, human osteoblasts, B16, TRAMP-C1 or MDA-231 MCs were obtained after 24 h of culture in 0% SFB α-MEM and were used to stimulate the different cell lines The pre-treated MCs were obtained after 24 h cultivation of the B16 or MDA-231 with MC of MLO-Y4, MC3T3-E1 or human osteoblasts followed by a wash with PBS and 24 h of culture with 0% SFB α-MEM. Finally, a cell lysate was obtained which was analyzed by PCR



been exposed to conditioned media of MLO-Y4 cells, caused an increase in the expression of RANK-L and a decrease in the OPG/RANK-L ratio. No changes were observed in the expression of OPG or Runx2 with respect to conditioned media without pre-stimulation (Figure 3A-D). These data suggest that melanoma tumor cells, which have been exposed to osteocyte secretoma, secrete soluble factors that cause a bone remodeling gene augmentation response, particularly increasing the expression of the osteoclastogenic factor RANK-L in osteocytes.

Interestingly, these effects did not reproduce in MC3T3-E1 osteoblastic cells, in which the conditioned media of B16 cells did not cause gene overstimulations (Figure 3E-H).

Communication by soluble factors between tumor cells of the breast and bone cells regulates the expression of remodeling genes in osteblasts

We then analyze the effects of cells of another type of tumor that metastasize to bone, such as breast cancer[5], on the gene expression of bone cells. In this case, we examine the effects of the secretome of the human tumor line of breast cancer MDA-MB-231 on human osteoblasts (Figure 1). The conditioned media of MDA-MB-231 cells was found to cause an increase in the expression of RANK-L and a decrease in the OPG/RANK-L ratio, without affecting the expression of OPG or Runx2 in human osteoblasts (Figure 3A -D). The effects on gene expression of osteoblasts were similar using the secretomes of MDA-MB-231, both pre-treated and not pre-treated with conditioned media of human osteoblasts (Figure 4A-C).

Communication through soluble factors between tumorous prostate cells and bone cells regulates the expression of remodeling and osteogenic genes in osteocytes

We then attempted to check if prostate tumor cells, potentially capable of metastasizing to bone[20,21], also established communication with bone cells through soluble factors (Figure 1). In this case, we observe that the secrets of TRAMP-C1 mouse prostate adenocarcinoma cells induced overexpression of the OPG and RANK-L genes, causing an increase in the OPG/RANK-L ratio, in addition to overexpressing the osteogenic transcription factor Runx2 in osteocytes MLO-Y4 (Figure 5A-D). Given

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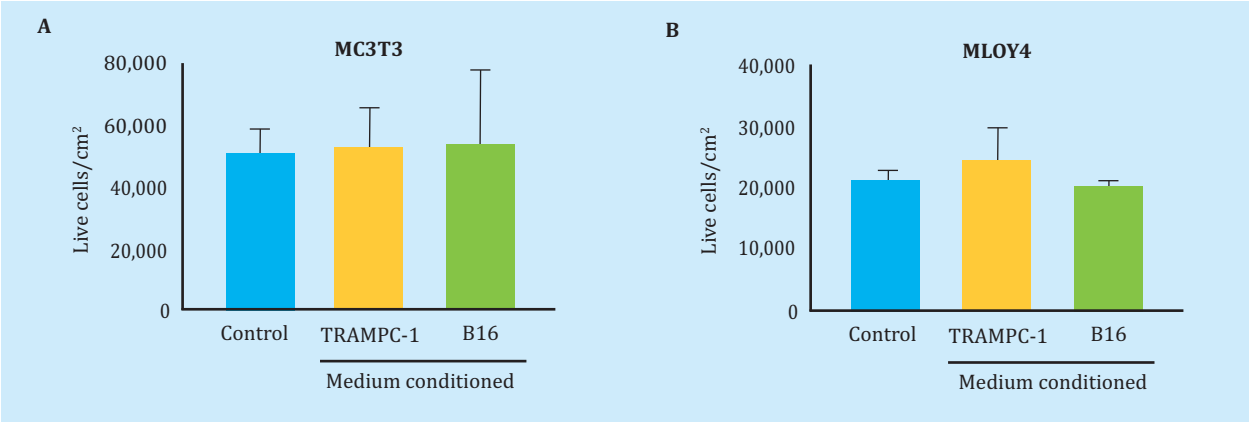
Table 1. Primers used in real-time PCR analysis

Gene	Sense (5´-3´)	Antisense (5´-3´)
RANK-L	TGTACTTTCGAGCGCAGATG	AGGCTTGTTTCATCCTCCTG
OPG	CAGAGCGAAACACAGTTTG	CACACAGGGTGACATCTATTC
Osteocalcin	GCAATAAGGTAGTGAACAGACTCC	CCATAGATGCGTTTGTAGGCGG
Runx2	CCTGAACTCTGCACCAAGTCCT	TCATCTGGCTCAGATAGGAGGG

Figure 2. Soluble factors secreted by mouse tumorigenic melanocytes (B16) or mouse prostate tumor cells (TRAMPC-1) do not affect the viability of pre-osteoblastic murine bone cells (MC3T3-E1) or osteocitic (MLO-Y4) data. Represented as the mean ± SEM of 2 experiments in triplicate



the gene overexpression of Runx2 in these cells, we wanted to check if the osteocalcin protein, a protein associated with bone formation and regulated by Runx2[22], also underwent changes in these conditions. Similar to Runx2, the expression of osteocalcin increased after stimulation with the secretion of TRAMP-C1 cells in MLO-Y4 osteocytes (Figure 5E).

Similar to the data observed with the secretomas of B16 melanoma cells, the conditioned media of TRAMP-C1 cells did not affect the gene expression of the markers previously mentioned in MC3T3-E1 osteoblasts (Figure 5F-J).

Discussion

The formation of a microenvironment that favors the implantation of circulating tumor cells was described by Kapplan. Their results demonstrated the formation of a pre-metastatic niche, in which a series of molecular and cellular changes were observed in the lung prior to the establishment of metastatic melanoma[15,23]. Other researchers subsequently described a series of sequential events that could involve the formation of a pre-metastatic niche in the liver, suggesting the involvement of exosomes derived from malignant pancreatic lesions as triggers of the process[14]. In this sense, little has been described about the formation of the pre-metastatic bone niche and the responses of the different bone subpopulations to the stimulation by secretomes of different tumors with high metastatic frequency to bone are unknown. In this article, we show for the first time how the secretome of solid tumors, with high organotropic potential in the formation of bone metastases, modifies the gene expression of genes related to bone metabolism in osteogenic lineage cells, and can be the triggering process in the induction of a favorable microenvironment for the settlement of tumor cells.

It has recently been suggested that primary tumor cells produce soluble tumor factors that trigger immature pre-metastatic niche formation[24]. Our results confirm that the secretomes of primary tumor cells (such as melanoma and prostate), or breast tumor cells derived from non-bone metastatic processes, mainly modify the balance between OPG and RANKL expression levels. This imbalance would decouple the relationship between osteoblastic bone formation and osteoclastic bone resorption, generating the release of growth factors and cytokines and thus initiating a "previous vicious cycle" of feedback that encourages the formation of future metastatic areas in bone[10]. In this sense, our results suggest the osteocyte as the cell most susceptible to soluble tumor factors. In our *in vitro* experimental model, in which we treat both osteocitic cells and mouse osteoblastic cells with conditioned media of murine cells of primary melanoma and prostate tumor, we observe significant changes in the gene expression of the osteocitic line without observing significant changes in the osteoblast line.

Given these observations, we conclude that, although both types of tumors will generate different types of metastases in the future, those derived from melanoma with a more osteolytic character[25] and those of preferably osteoblastic prostatic adenocarcinoma, the initial stages, in which alterations occur in bone cell physiology, are common. These observations suggest that the modifications that originate in the pre-metastatic niche do not predispose the specific type of skeletal lesions that will develop in metastatic disease.

Figure 3. Soluble factors secreted by tumorgenic melanocytes (B16) modify gene expression of bone remodeling in osteocytes (MLO-Y4) but not in pre-osteoblasts (MC3T3-E1). The expression of OPG (A, E), RANK-L (B, F), OPG/RANK-L ratio (C, G) and Runx2 (D, H) was evaluated by real-time PCR after stimulating 24 h MLO-Y4 and MC3T3-E1 with conditioned medium of B16 pre-treated or not. Data are represented as the mean ± SEM of three independent experiments in triplicate. *p<0.05 vs control, **p<0.01 vs control

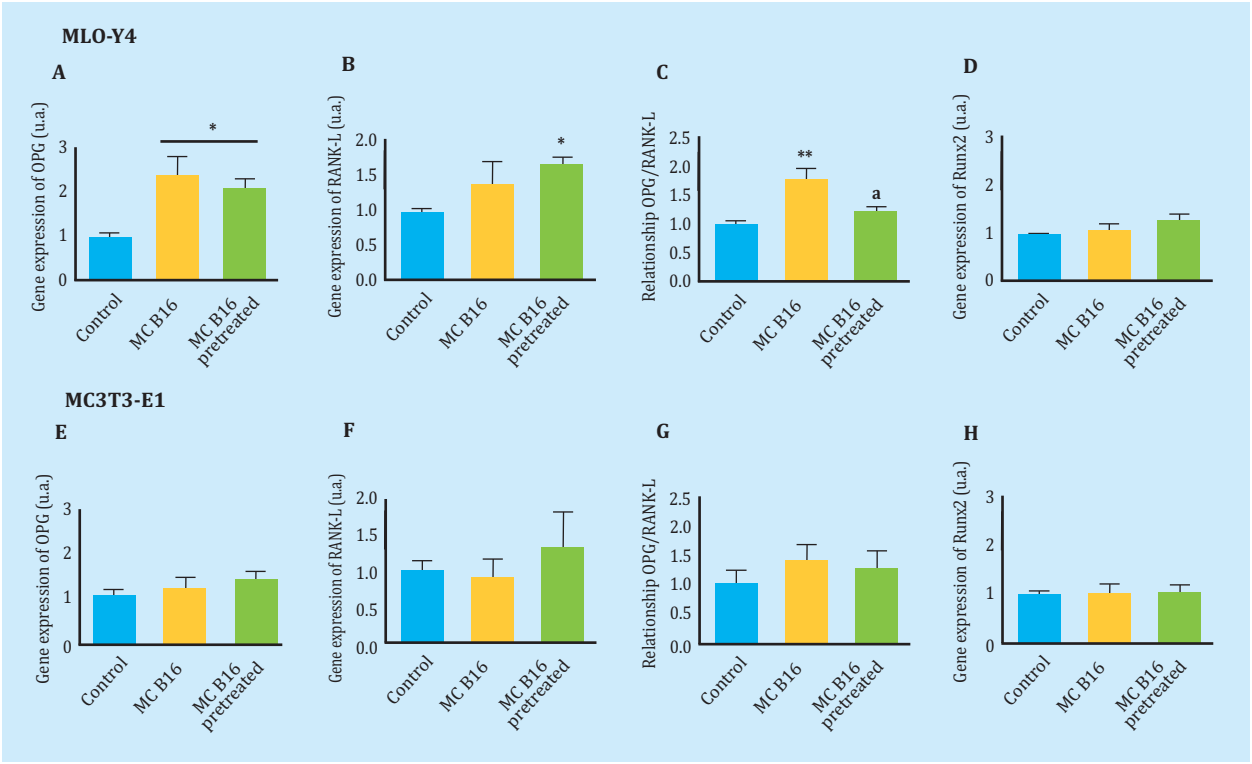


Figure 4. Soluble factors secreted by breast cancer tumor cells (MDA-MB-231) modify bone remodeling gene expression in human osteoblasts (hFOB 1.19). The expression of OPG (A), RANK-L (B), OPG/RANK-L (C) and Runx2 (D) ratio was evaluated by real-time PCR after 24 h stimulation of human osteoblasts with conditioned medium of pre-MDA treated or not. Data are represented as the mean ± SEM of three independent experiments in triplicate. *p<0.05 vs control

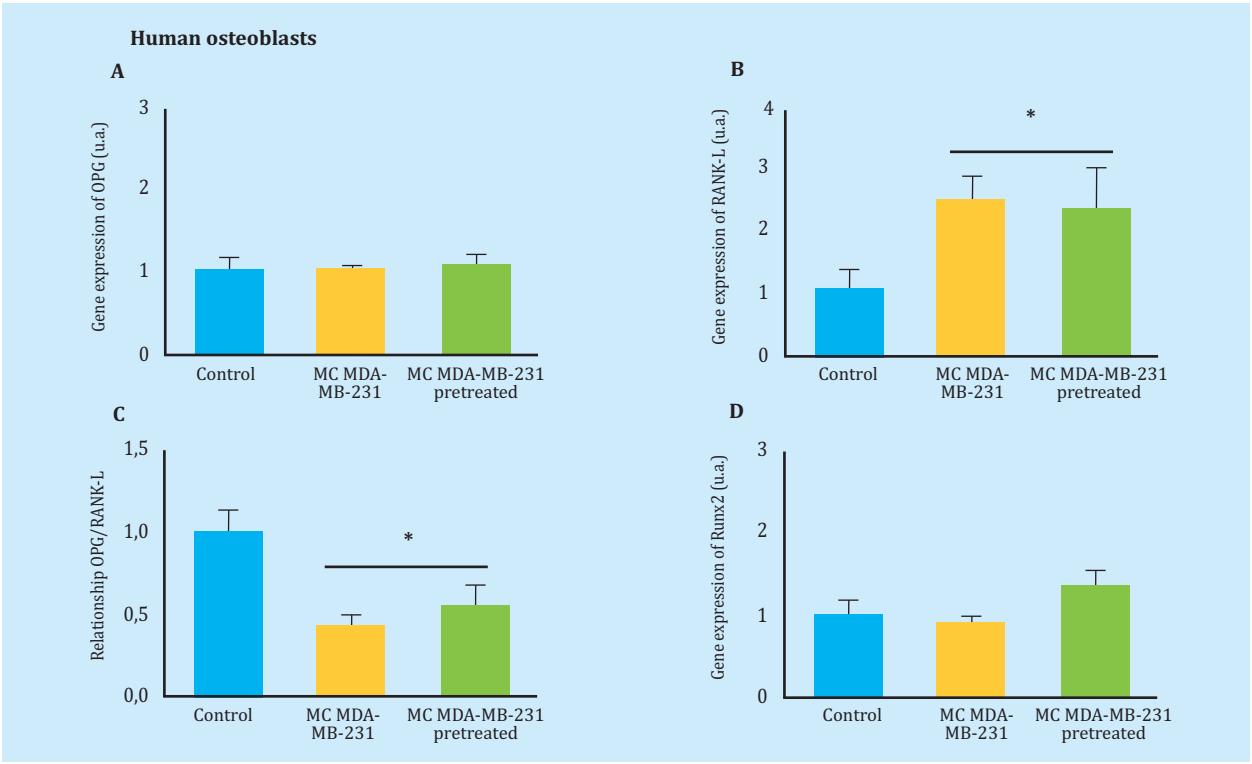


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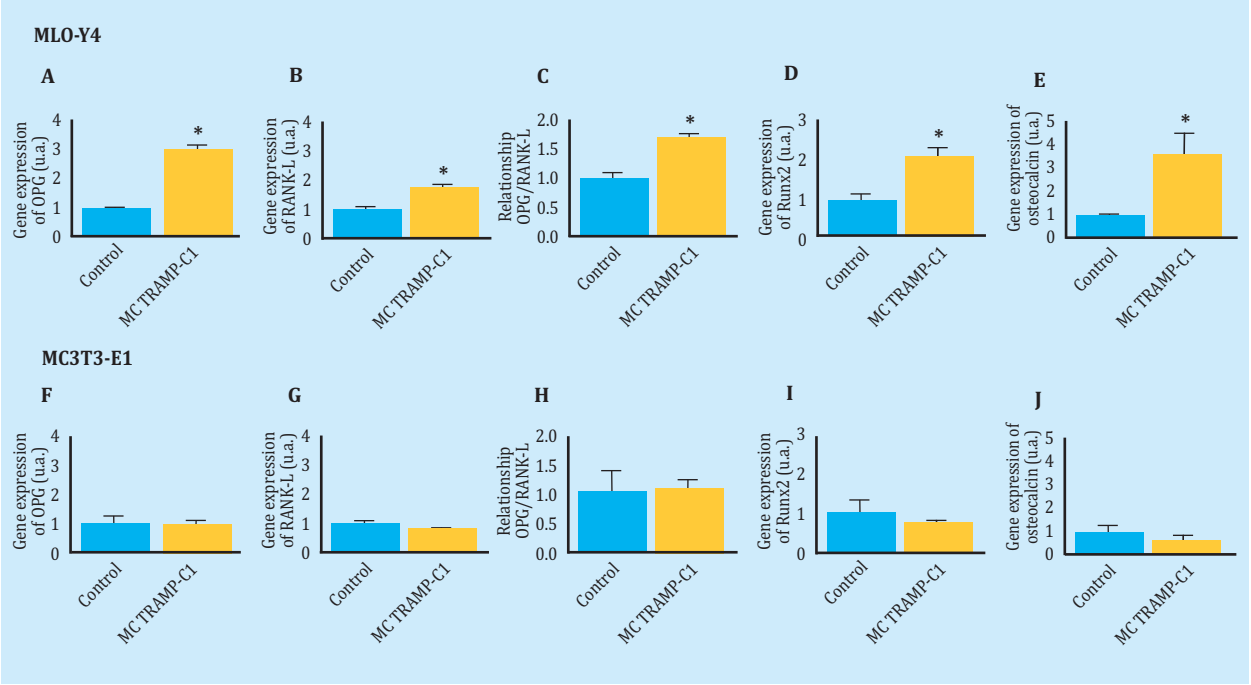
Figure 5. Soluble factors secreted by prostate tumor cells (TRAMP-C1) modify the expression of bone remodeling genes in osteocytes (MLO-Y4) but not in pre-osteoblasts (MC3T3-E1). The expression of OPG (A, F), RANK-L (B, G), OPG/RANK-L ratio (C, H), Runx2 (D, I) and osteocalcin (E, J) was evaluated by time PCR after stimulating 24 hours MLO-Y4 and MC3T3-E1 with conditioned medium of TRAMP-C1. Data are represented as the mean ± SEM of three independent experiments in triplicate. *p<0.05 vs control

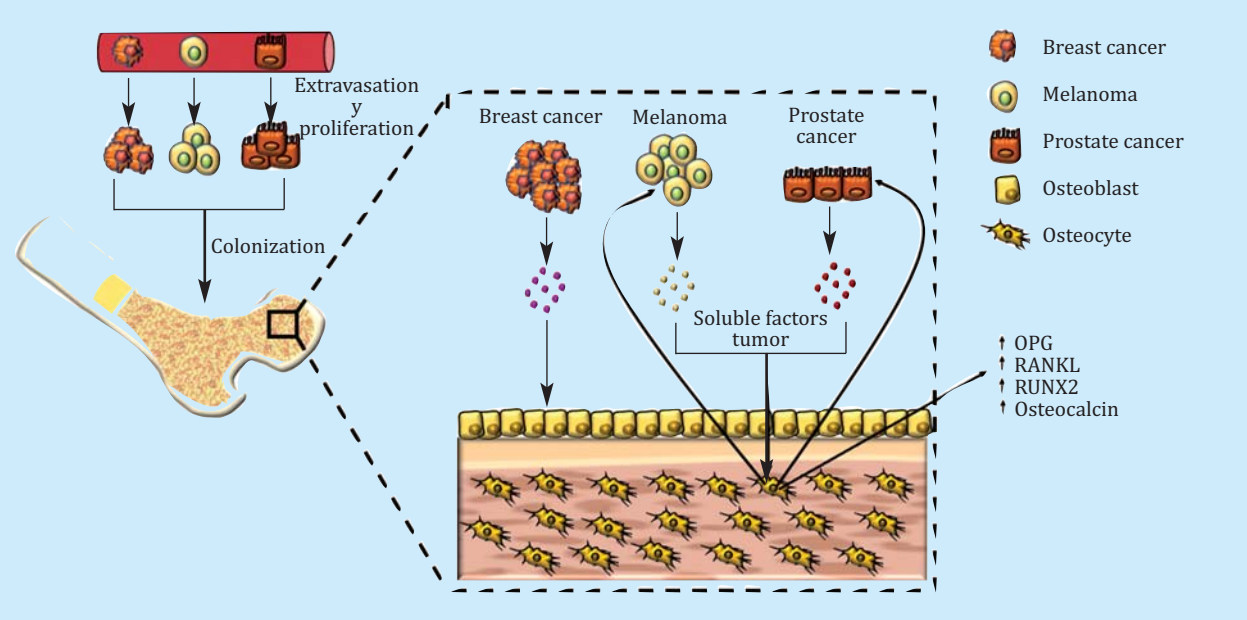


Notably, we suggest osteocyte as the main target cell of soluble tumor factors. Since there is no human osteocitic cell model of continuous lineage, we could not corroborate the results obtained by the melanoma and prostate cancer secretomes compared to the breast cancer secretomes in these cells.

The study of the changes induced by the secretome of breast cancer cells on bone cells was limited to the effects on the human osteoblastic continuous line hFOBs. The results obtained suggest that breast cancer cells are capable of affecting osteoblasts by soluble factors and that they mainly modulate the gene expression of RANK-L without the need to maintain cross-communication with these cells, confirming the alteration of the OPG/RANKL axis, as well as the changes induced by the other tumor secretomes studied. Given that osteocytes are the majority cells in bone, with a half-life of 25 years, with a multifunctional character and role of director-coordinator of the bone environment[26,27], their modulation, either pharmacological or as a therapeutic target, could be key to avoid pre-metastatic niche formation and bone metastasis. It has been proposed that osteocytes, and not the rest of the cells of the osteogenic lineage, are the main source that the osteoclasts require for their formation and activation in the remodeling of the trabecular bone[28,29], and may also be the only ones responsible for initiating the vicious cycle of the bone metastatic process. Few studies have established a clear role of the osteocyte and its relationship with cancer. In this sense, Delgado-Calle et al. showed that the osteocytes could be regulators of the proliferation of myeloma-like cancer cells by direct interaction with them through their cytoplasmic prolongations, which are capable of reaching the periodic and endocortical surfaces, as well as the bone marrow surface[30]. This direct interaction would lead to activation of the Notch signaling pathway in myeloma cells. This pathway mediates cell-to-cell communication, and participates in the control and activation of cell proliferation and death programs. Its pharmacological inhibition, using an inhibitor of said pathway, prevented the proliferation of myeloma induced by osteocytes[30]. In addition to regulating and coordinating the rest of cell lines in the bone environment, the osteocytes could secrete factors that reach distant cells, such as, for example, primary tumor cells, and modify them. This communication has been suggested in the case of prostate adenocarcinoma, where osteocitic and osteoblastic cells regulated their osteomimetic properties[21]. In addition, this communication could modulate key signaling pathways in prostate tumor cells, such as calcium intracellular mediators, cyclic AMP and ERK ½[31], and may enhance tumor progression of prostate cancer to bone. All these results, together with our results shown in this paper, suggest that in the cross-communication between the primary tumor and bone cells, the composition of the factors involved in the secretome may vary. During the evolution of the tumor, soluble tumor factors could reach the bone environment and induce changes in their cells. These cells could also send soluble factors to the primary tumor, which will generate changes in the tumor cells inducing them a greater osteomimetic phenotype.

The secretome of these osteomimetic tumor cells (in the figures presented as pre-treated conditioned media), upon reaching the bone environment again, could affect in the same direction or induce different variations of the changing microenvironment towards the formation of the niche. In this sense, in the proteomic analysis of the secretome of two continuous prostate tumor lines with different origin, DU145 (from brain metastases) and PC-3 (from bone metastases) 211 differentially expressed

Figure 6. Tumor secretomes modify the bone environment, the osteocytes being the most sensitive cells to these changes. The soluble factors secreted by prostate and melanoma tumors are those that mainly affect the osteocyte, increasing the gene expression of different factors related to bone remodeling. In turn, the osteocytes secrete factors that modify and induce a greater osteomimetic phenotype in the tumor cells themselves



secretion proteins were found, which meant a 37.6% of the total proteins analyzed, indicating that the secretion proteins were considerably different between both cell lines[32]. Based on these investigations and our results, we propose that tumor secretomes modify the bone environment, the osteocytic line being more sensitive (Figure 6). The results shown open a wide field of study in the knowledge of the communication between the primary tumor cells and the cells of the future metastatic organ, in this case, the bone microenvironment. However, they should deepen their knowledge, due to the limitations of the present study. As previously mentioned, nonspecific effects have been avoided as a result of using cell cultures of different species, and an *in vitro* cell model based on widely used cell lines has been used as a reference for the study of bone metabolism and physiology, in the case of the MC3T3, MLOY-4 and hFOBs; as well as in the study of cancer, in the case of prostate tumor cells, melanoma or breast. With respect to the breast tumor line (MDA-MB-231 HTB-26), one of the limitations of its use in *in vitro* models is its metastatic origin[33], its behavior can be seen as a primary tumor and with it metastatic organotrophic communication .

Thus, the other two tumor lines with bone metastatic organotropism whose origin comes from primary tumors have been studied. However, it would be advisable to confirm the results obtained in this work in an *in vivo* experimentation model where you can study and confirm the changes in the bone microenvironment prior to the establishment of the metastasis, quantifying in the serum/plasma the altered bone cytokines and determining the temporality of bone and primary tumor communication as well as the level of change in bone tissue. The more detailed knowledge of the molecular changes that involve the formation of the pre-metastatic bone niche, as well as the secretory factors that induce it, could provide new therapeutic targets or action protocols, thus improving its prognosis. These actions would reduce the metastatic skeletal events developed by patients with solid tumors such as prostate or breast tumors, thus increasing their quality of life.

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Conflict of interests: The authors declare no conflict of interest.

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Metastatic transverse vertebral fracture due to lung cancer

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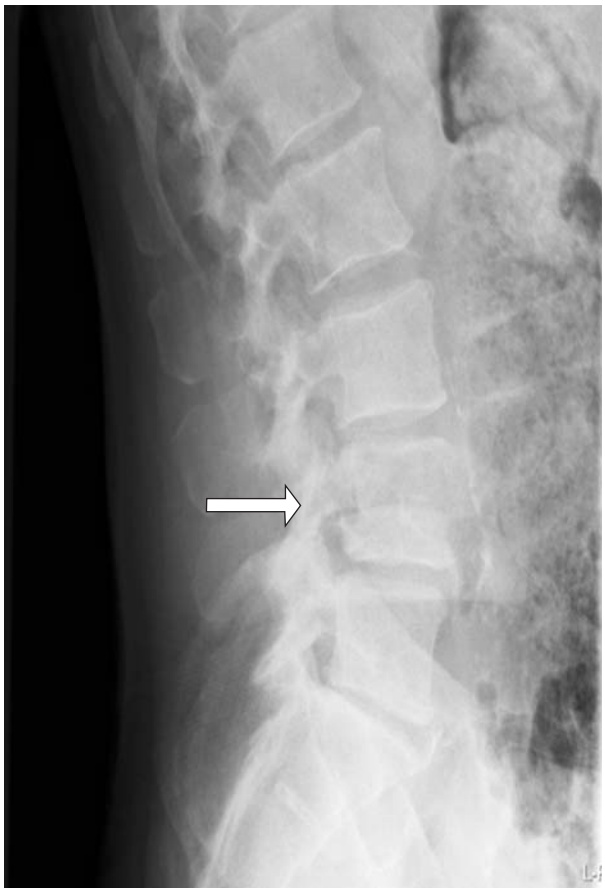
A 58-year-old patient with rheumatoid arthritis in remission with methotrexate at a dose of 10 mg/week. He goes to hospital emergencies several times for acute lower back pain over one month. In the lumbar X-ray, an L4 transverse fracture with posterior wall retropulsion (Figure 1) goes unnoticed. This lower back pain becomes disabling with loss of left leg function. Lumbar MRI is carried out on T2 and STIR sequence (Figures 2a and 2b), showing acute-subacute fracture of the L4 vertebral soma with pedicles edema and moderate intra-canal displacement of the lower half of the posterior wall that compresses the efferent nerve root. Left and partially takes up the side recess. With suspicion of tumor etiology, enter for study. In the thoracic CT scan, a large, right-lobed, upper-cavity tumor is reported with ipsilateral main bronchus associated with perilesional pneumonitis and bronchiectasis (Figure 3). The pulmonary lesion histology was of large cell lung carcinoma PD-L1 80% positive. Vertebroplasty was carried out. The patient underwent pembrolizumab treatment with good response to date. Rheumatoid arthritis is maintained in remission despite treatment with anti-PDL[1].

We present a case of transverse vertebral fracture secondary to metastasis from lung cancer. These fractures are very rare and often go unnoticed. They may appear in patients with ankylosing spondylitis and vertebral fusion, but outside this context they tend to suggest an underlying neoplastic growth. The main tumors to consider are myeloma or metastases of prostate, breast or lung. There are other less frequent primary tumors such as kidney, colon, skin or thryroids[2,3].

Figure 1. Lateral lumbar radiography. An L4 transverse fracture with posterior wall retropulsion is observed



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Figures 2a and 2b. Lumbar NMR in T2 and STIR that shows acute-subacute fracture of the L4 vertebral soma with pedicles edema and moderate intra-canal displacement of the lower half of the posterior wall that compresses the left efferent nerve root and partially occupies the lateral recess

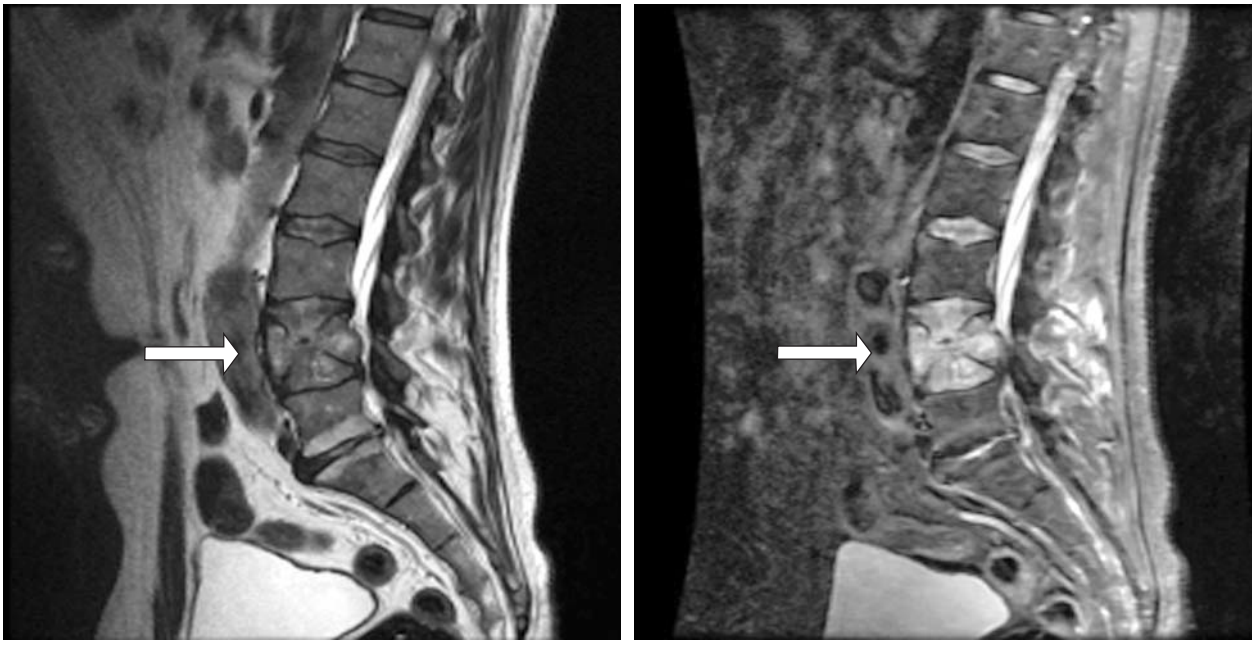
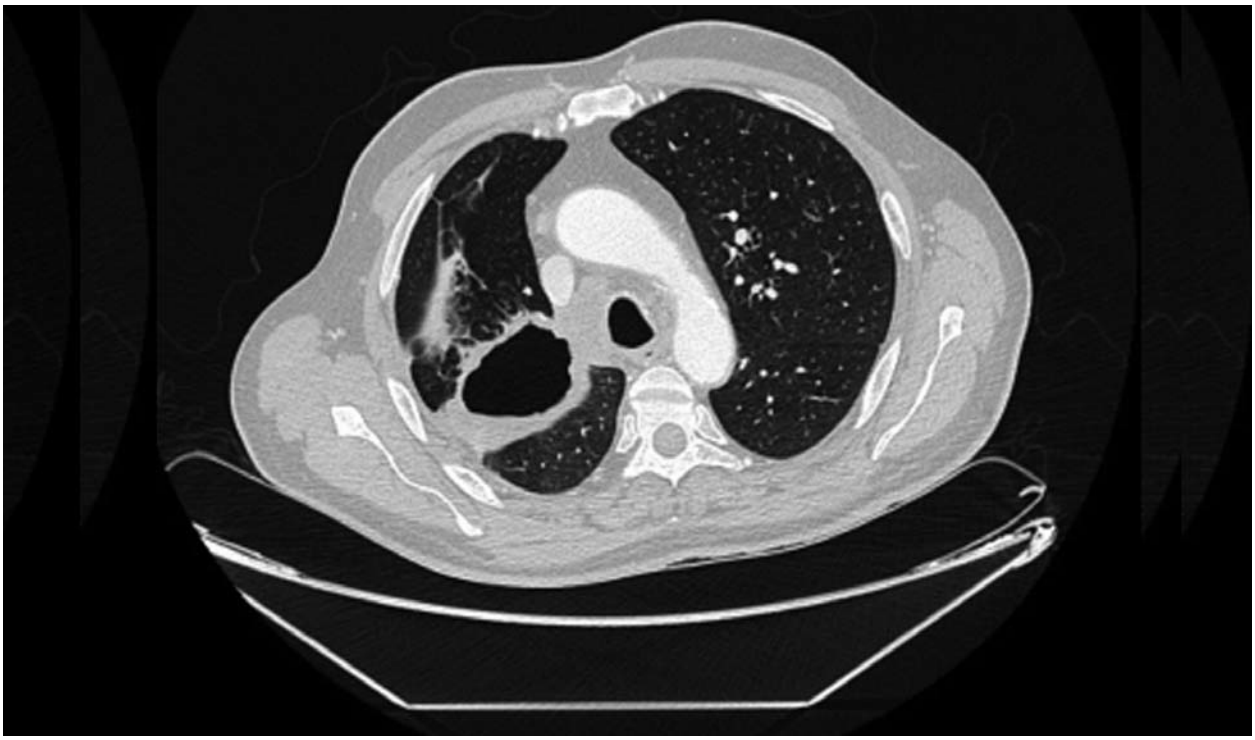


Figure 3. Chest CT scan shows a large lesion in the right upper lobe



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Vitamin D and heart failure. Pathophysiology, prevalence, and prognostic association

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Introduction

Heart failure (HF) is a major public health problem characterized by high mortality, frequent hospitalizations and deterioration in the quality of life, with a prevalence and incidence that is increasing worldwide[1,2]. Although the prognosis has improved in recent decades thanks to the diagnostic and therapeutic improvement of cardiovascular diseases, the morbidity and mortality of these patients remains high[3]. All this implies that new objectives and treatment options are still needed.

Vitamin D had traditionally been associated only with bone health, accepting that vitamin D deficiency caused osteomalacia and osteoporosis in adults and rickets in children[4,5]. However, data obtained in recent years indicate that vitamin D is an important micronutrient for optimal function of many organs and tissues throughout the body, including the cardiovascular system[6,7]. It has been suggested that vitamin D deficiency may be an important factor both in the genesis of risk factors and cardiovascular disease[7] as a prognostic marker in HF. Pathophysiological data indicate that vitamin D deficiency may be very harmful for patients with HF and that vitamin D supplementation can be potentially beneficial, although all this is not without controversy[8].

In this paper, we review the evidence that so far supports the link between vitamin D and HF. We analyze the potential mechanisms through which vitamin D could exert, its cardioprotective effects, the potentially deleterious effects of its deficit and break down the main studies on vitamin D supplementation in patients with HF.

Pathophysiology of vitamin D and heart failure

There is no single established route or a single hypothesis that explains the relationship between vitamin D and HF. The vitamin D receptor (RVD) is a nuclear hormonal receptor that mediates the action of calcitriol through genomic and non-genomic pathways[9]. Cardiomyocytes have RVD, and it is known that calcitriol through RVD also modulates important genes related to cardiovascular health, so they may be influenced by vitamin D[10].

Functional RVDs are expressed in the cell nucleus or adjacent to the T tubules of cardiomyocytes, and also of cardiac fibroblasts. Cardiac hypertrophy has been associated with an increase in the expression of these receptors in these cells. Vitamin D has also been attributed an antiproliferative property mediated by the suppression of proto-oncogenes such as c-mic, as well as the natriuretic peptide, acting directly on the growth and differentiation of cardiomyocytes. Mice without RVD (knock-out of the VDR gene) show a greater deposition of collagen in their cardiac structures[10].

There are also more complex molecular mechanisms that can explain the relationship between vitamin D and HF. Vitamin D acts on the calcium channels of the cardiomyocytes inducing a rapid intracellular calcium influx[11]. This intracellular calcium concentration controls long-term growth, proliferation and cell death responses. In addition, by activating the protein C-kinase, it promotes cardiomyocyte relaxation and, therefore, participates in cardiac diastolic function[12] and in cardiac systole through activation of adenyl cyclase or cyclic adenosine monophosphate. Dysfunction of any of these pathways can cause systolic and/or diastolic ventricular dysfunction, and, therefore, HF.

Several neuroendocrine systems and inflammatory cytokines are involved in the pathophysiology of vitamin D and HF. These are activated to maintain circulatory homeostasis, but in the long term they contribute to increased systemic resistance and ventricular remodeling, developing and worsening HF. Although the renin-angiotensin aldosterone system (SRAA) and the sympathetic nervous system (SNS) have so far been the most important in HF, recently, both at the diagnostic and therapeutic level, counter-regulatory systems such as natriuretic peptides are also being key. in the diagnostic-therapeutic approach of this syndrome[13,14].

It has been shown that vitamin D has an intimate relationship with SRAA. Different studies have shown that there is an inverse correlation between vitamin D levels and the activity of SRAA[15-17]. The main actions of SRAA include the regulation of vascular tone, volemia, ventricular and vascular remodeling and activation of SNS. The key role of SARS in the pathophysiology of HF and arterial hypertension is well defined.

The cascade of action and pathophysiology of SRAA is as follows: renin is a protein that acts on angiotensinogen, producing angiotensin I, which is transformed into angiotensin II by the action of angiotensin converting enzyme at the pulmonary and vascular level. Angiotensin II is a potent vasoconstrictor hormone of afferent and efferent renal arterioles, and also promotes the activation of the sympathetic nervous system (also key in

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the pathophysiology of HF). The overactivation of SNS and SRAA contributes to the progressive cardiac remodeling that can lead to HF. This hormone in turn also promotes the release of aldosterone from the adrenal cortex, important in electrolyte and volume balance by retaining sodium and water and releasing potassium and magnesium at the renal level[18].

Although one of the main factors that stimulate the release of renin, and therefore SRAA, is the decrease in renal perfusion, experimental studies have shown that after modifying the RVD function in experimental animals with knock-out RVD mice, an increase in the concentration of renin expression with increased mRNA and its protein in the kidney, and plasma angiotensin II, compared to wild-type mice[15]. Consequently, these knock-out mice developed more arterial hypertension, left ventricular hypertrophy and an increase in fluid retention. Injection of 1.25 (OH) $_2$D achieved marked suppression of renin, which was also achieved with the use of the angiotensin-converting enzyme inhibitor, captopril, or angiotensin II receptor antagonist, losartan; which showed the key pathophysiological role of SRAA[15,19], demonstrating that the probable cause of this is overstimulation of SRAA[20]. The role of angiotensin II in increasing fibrosis and cardiac hypertrophy, increasing vascular tone and therefore blood pressure, as well as increasing sympathetic tone, and direct relationship with the symptoms and progression of heart failure in humans, is also clearly established.

Vitamin D deficiency has been linked to an increase in the production and release of inflammatory cytokines, which has an indirect and direct negative effect on the heart and other organs. Inflammatory cytokines induce apoptosis of cardiomyocytes, hypertrophy, fibrosis, cardiac remodeling and negative ionic alterations such as sodium retention and, therefore, fluid retention[21]. It also increases catabolic activity and induces cachexia, which contributes to the progression of HF syndrome[22]. *In vitro* studies have suggested that vitamin D inhibits inflammatory cytokines such as TNF-α and IL-6, while stimulating anti-inflammatory cytokines such as IL-10[23].

RVDs are also present in the parathyroid gland, and calcitriol suppresses the production of parathyroid hormone (PTH) and prevents the proliferation of parathyroid glands[24]. When there is a vitamin D deficiency, secondary hyperparathyroidism occurs, which also has deleterious cardiovascular and trophic effects on cardiomyocytes. This increase in PTH levels also leads to an increase in blood pressure due to an increase in arterial stiffness and, therefore, and once again, contributes to cardiac remodeling in HF secondary to hypertrophy, apoptosis and fibrosis of the ventricle[10,25].

Another pathophysiological mechanism is the influence of vitamin D on the regeneration of the myocardial extracellular matrix, another route by which it can be harmful for cardiac structure and function. Experimental studies with knock-out RVD mice have shown that the absence of vitamin D is associated with an increase in the expression and activity of myocardial matrix metalloproteinases (MMP), which results in myocardial remodeling, increased collagen deposition and greater fibrosis[26,27]. Vitamin D modulates the regeneration of the extracellular matrix of the myocardium by acting on the expression of both matrix metalloproteinases (MMPs) that hydrolyse extracellular matrix (ECM) proteins and tissue inhibitors of metalloproteinases (TIMP). In knock-out RVD mice, the unbalanced expression of MMP/TIMP was characterized by the positive regulation of the MMP-2 and MMP-9 negative metalloproteinases of TIMP-1 and TIMP-3. The imbalance between MMP and TIMP promoted the destruction of myocardial tissue and ventricular remodeling; All this is closely related to the complex processes of initiation and progression of diastolic and systolic HF[28]. It should also be noted that certain inflammatory cytokines such as TNF-alpha are also an important regulator of MMP activity, and can contribute to this pathophysiological pathway[29].

Coronary artery disease is an important factor for the development of HF, and vitamin D deficiency has been associated with an increase in arteriosclerosis and calcification of the coronary arteries[1,9,27,30]. This observation is consistent with the inverse objectified relationship between vitamin D levels and coronary artery calcification[6,30,31]. It is documented that endothelial cells also express RVD, and that vitamin D increases nitric oxide activity *in vitro*[32], improves vascular endothelial growth factor production[33] and reduces endothelial platelet aggregation[34]. Finally, there is evidence that vitamin D deficiency may be an important regulatory factor of the cardiorenal system. As we have previously emphasized, the cardiovascular and renal system are closely related, so that alterations in the functioning of one can progressively deteriorate the other[34].

When there is a progression of cardiorenal syndrome, this also implies neurohormonal activation, mainly of the renin-angiotensin system and the sympathetic nervous system, and of inflammatory systemic mechanisms as described previously. This once again influences fibrosis and ventricular remodeling, hydroelectrolytic abnormalities, and cardiac and renal dysfunction; triggering a negative vicious circuit in response to deterioration of the cardiorenal system, with greater neurohormonal activation and inflammatory cytokines, resulting in greater systemic dysfunction.

In the population with chronic kidney disease (CKD), as in the population with HF, the prevalence of hypovitaminosis D is high, and has also been associated with an increased risk of cardiovascular events[34]. A reduction in enzyme 1-alpha-hydroxylase activity and the depletion of vitamin D binding proteins to RVD secondary to proteinuria are responsible for patients with CKD having a vitamin D deficiency. Therefore, the close correlation of HF and chronic kidney disease highlights the importance of vitamin D in both pathologies and in the pathophysiology of cardiorenal syndrome.

Prevalence of hypovitaminosis D and heart failure

Although there is no consensus on the optimal levels of vitamin D, the deficiency of this hormone is defined by most experts as a level of 25-hydroxy-cholecalciferol (25-HCC) below 20 ng/ml[4,38-42]. To be more specific, according to the consensus uniformly accepted by scientific societies dedicated to bone mineral metabolism, patients are considered to have optimal levels of vitamin D when serum 25-HCC levels are above 30 ng/ml; between 29 and 20 ng/ml it is considered that there is an insufficiency; and below 20 ng/ml the existence of a deficiency is established, which would be severe with 25-HCC figures below 10 ng/ml[38,41]. Likewise, the theory of the U-shaped relationship between vitamin D levels and any cause of mortality, cardiovascular disease, certain types of cancer, falls and fractures has emerged, and that

Table 1. Prevalence of vitamin D deficiency in heart failure (HF)

First author Year Type of study	Patients (n)	Inclusion criteria	Definition of hypovitaminosis	Average age (years) /women (%)	Prevalence
Gostman I *et al.* 2012 Prospective	3,009/46,825	Patients ≥45 years with HF *vs.* control population with 25-HCC measure	25-HCC <10 ng/ml	75.9±10.7/ 64.7/11.3	14.8 ng/ml/16.3ng/ml (28% *versus* 22%, p<0.00001). In HF group, only 8.8% had 25-HCC >30 ng/ml
Liu L *et al.* 2011 Prospective	548	HF NYHA II-IV	25-HCC <20 ng/ml	74/61	75% cohort
Kim DH *et al.* 2008 Cross	8,351	Adults with 25-HCC levels measured	25-HCC <30 ng/ml		74% of the general population. 89% of hypovitaminosis D in patients with HF and ACS
Zittermann *et al.* 2003 Cross	24/34/34	HF patients NYHA II-IV <50 years *vs.* ≥50 years vs. control without HF ≥50 years		38.9/64.1/68.9	Both groups with HF had decreased levels of 25-HCC and calcitriol (p<0.001). Reverse correlation of vitamin D levels with severity of HF (assessed by levels of Nt-proBNP)
Shane E *et al.* 1997 Cross	101	HF NYHA III-IV; transplant consideration	25-HCC ≤9 ng/ml	NE/22	17% of hypovitaminosis D in patients with 25-HCC ≤9 ng/ml and 26% with 1.25 $(OH)_2D$ ≤15 pg/ml. Inverse correlation between severity of HF and vitamin D levels

NYHA: New York Heart Association scale; ACS: acute coronary syndrome; Nt-proBNP: N-terminal cerebral natriuretic propeptide; NE: not specified.

vitamin D poisoning is observed with serum levels 25-HCC >150 ng/ml[43]. Clinical practice guidelines recommend that plasma vitamin D levels should not be routinely measured in the general population and should only be measured in patients from populations considered at risk for this hormone deficiency[44-47].

In recent years it has been shown that vitamin D deficiency has probably been underestimated and is much more prevalent than had been recognized. A global prevalence of one billion individuals with deficit and insufficient vitamin D levels is estimated[42] and it has been described that 40-80% of the adult population has a vitamin D deficit[48,49], being of special importance in women in Middle Eastern countries. Numerous risk factors for vitamin D deficiency have been described, such as age, skin hyperpigmentation, hospitalization in institutions, distant latitude of Ecuador, obesity, smoking, nephropathy, hepatopathy or certain drugs such as corticosteroids, phenytoin or phenobarbital[49]. Of particular relevance is the global obesity epidemic in developed countries, which significantly influences the deficit of vitamin D, given the kidnapping that occurs of this hormone in the adipose tissue[48].

It is also true that a vitamin D deficit has been observed in both the young and apparently healthy population[50], described in up to approximately 50% of young adults; even in studies carried out in areas with high exposure to sunlight such as the Canary Islands, Israel, Australia, Turkey, India or Lebanon, where 30-50% of children and adults have 25-HCC levels <20 ng/ml[51-53].

Likewise, data obtained from the National Health and Nutrition Examination Survey (NHANES) showed a prevalence of hypovitaminosis D of 74% in the general population, significantly increasing the prevalence to 89% when only patients with HF and coronary artery disease were considered. (odds ratio [OR]=3.52; 95% confidence interval [CI]: 1.58-7.84)[54]. Another study, with similar characteristics in which 4,105 subjects from a general population with at least one determination of vitamin D were included, found that only 36% of this cohort had vitamin D levels within normal range. A higher prevalence of heart failure (90% relative and 9% abso-

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Table 2. Vitamin D levels and risk of heart failure (HF)

First author Year Type of study	Patients (n)	Inclusion criteria	Average age (years) Sex F (%)	Follow-up	Results
Bansal N *et al.* 2014 Prospective	6,469	General population free of established CVD (Multi Ethnic Study of Atherosclerosis)	62 53	8.4 years	- Comparison between patients with PTH <65 and ≥65 pg/ml; the latter had 50% (95% CI: 3-20%) higher risk of incidence of HF and 5.3 g (95% CI: 2.6-7.9 g) plus left ventricular mass due to MRI - No association between HF and 25-HCC
Wannamethee SG *et al.* 2014 Prospective	3,713	General population aged 60-79 years with and without established CVD	68 0	13 years	-In patients with PTH >55.6 pg/ml, risk of de novo CI of 1.66 HR (95% CI: 1.30-2.1) - No association between 25-HCC, calcium or phosphorus levels with a risk of CI (HR=1.07; 95% CI: 0.67-1.71)
Kestenbaum B *et al.* 2011 Prospective	2,312	Healthy subjects ≥65 years	75 NI	14 years	- In patients with 25-HCC <15 ng/ml, risk greater than 29% (95% CI: 5-55% higher) of mortality from any cause. Every 10 ng/ml less than 25-HCC was associated with a 9% higher relative risk of mortality (95% CI: 2-17% higher) - In patients with PTH ≥65 pg/ml, a risk greater than 30% (95% CI: 6-61%) of incidence of HF
Pilz S *et al.* 2008 Prospective	3.299	Caucasian patients undergoing cardiac catheterization	63 NI	7.7 years	2.84 HR (95% CI: 1.20-6.74) for death by CI and 5.05 HR (95% CI: 2.13-11.97) for sudden death when comparing patients with severe hypovitaminosis D (25-HCC <10 ng/ml *versus* patients with optimal levels of vitamin D (25-HCC >30 ng/ml). Inverse correlation with 25-HCC and 1.25 (OH) 2D level levels, and Nt-proBNP (r=-0.190 and -0.255, respectively; p<0.001) and with LVEF (p<0.001)

CVD: cardiovascular disease; NMR: nuclear magnetic resonance; NI: not indicated; HR: hazard ratio; CI: confidence interval; Nt-proBNP: N-terminal cerebral natriuretic propeptide; LVEF: left ventricular ejection fraction

lute) was observed in subjects with vitamin D levels ≤15 ng/ml, and in a follow-up of 1.3±1.2 years of a population over 50 years an incidence of new cases of HF was observed in 2.5% of this cohort. In this study, plasma vitamin D levels was found to have an inverse correlation with HF risk, and the adjusted risk values for HF were 2.01 and 1.3 for values between 16-30 ng/ml. and ≤15 ng/ml, respectively[7].

A high prevalence of vitamin D deficiency has also been demonstrated in patients with HF evaluated for heart transplantation, as well as its inverse correlation between serum levels of vitamin D and the severity of HF[55]. This relationship has also been objectified by other groups using controls without heart failure[56] even in younger patients, which suggests that there is an association between HF and vitamin D deficiency that is independent of age.

Therefore, although there is a high prevalence of hypovitaminosis D in the apparently healthy general population, this deficit seems to be more marked in the population with HF (Tables 1, 2 and 3).

Relationship between vitamin D and heart failure

HF is a disease with a high socio-sanitary impact, so a special effort has been made in predicting the risk of developing HF and identifying that population at risk in which a more active primary prevention should be emphasized. Therefore, and given the high morbidity and

Table 3. Prognostic impact of vitamin D deficiency in patients with heart failure (HF)

First author Year Type of study	Patients (n)	Inclusion criteria	Follow-up	Levels of vitamin D	Results
Gruson D *et al.* 2015 Prospective	170	HF NYHA II-IV with LVEF ≤35%	4.1 years	NE	- Inverse correlation of levels of 1.25 $(OH)_2D$ with the severity of CI (mean values: NYHA II: 33.3 pg/ml, NYHA III: 23.4 pg/ml, NYHA IV: 14.0 pg/ml; p<0.001) - 1.25 $(OH)_2D$ and its ratio to PTH (1-84) independent predictor of cardiovascular mortality in HF
Gostman I *et al.* 2012 Prospective	3,009 with HF /46,825 control	Patients ≥45 years with HF *vs.* control population with 25-HCC measure	518 days	25-HCC <10 ng/ml	- Higher mortality in patients with HF (HR=1.52; 95% CI: 1.21-1.92; p<0.001) - Reduction of mortality in patients who received vitamin D supplementation (HR 0.68; 95% CI: 0.54-0.85; p<0.0001
Liu L *et al.* 2011 Prospective	548	HF NYHA II-IV hospitalized	18 months	NE	- For every 4 ng/ml decrease in 25-HCC, increased risk of death due to combined final objective of death from any cause or heart failure and rehospitalization (HR=1.09; 95% CI: 1.00-1.16) and higher mortality from any cause (HR=1.10; 95% CI: 1.00-1.22) - No significant effect on HF rehospitalization

NYHA: New York Heart Association scale; LVEF: left ventricular ejection fraction; HR: hazard ratio: CI: confidence interval; NE: not specified.

mortality of HF, it is interesting to find prognostic markers in this pathology that can predict mortality. In this regard, attempts have also been made to confirm the association between vitamin D levels and the risk of HF and adverse events through longitudinal studies.

Vitamin D has been associated with the development of HF and as an independent prognostic factor for HF mortality and sudden death in a prospective study of 3,299 Caucasian patients undergoing cardiac catheterization, with a follow-up of 7.7 years on average. When patients with severe hypovitaminosis D (25HCC <10 ng/ml) were compared with patients with optimal levels of vitamin D (25-HCC >30 ng/ml), a hazard ratio (HR) of 2.84 (CI) was obtained. 95%: 1.20-6.74) for death due to HF and 5.05 (95% CI: 2.13-11.97) for sudden death. There was also an inverse correlation between levels of N-terminal cerebral natriuretic propeptide (Nt-proBNP) and serum vitamin D levels, and an inverse association with the NYHA functional class (New York Heart Association)[57]. These findings have also been subsequently corroborated in a study of 2,312 healthy subjects over 65 years of age, where it was observed that patients with 25-HCC <15 ng/ml had a risk greater than 29% (95% CI: 5-55% higher) of mortality from any cause, and that for every 10 ng/ml that decreased 25-HCC increased the relative risk of mortality by 9% (95% CI: 2-17%)[20].

To date, data that relate vitamin D levels to the risk of developing HF are discrepant. On the one hand, an unequivocal association of vitamin D levels with the incidence of HF has not been objectified, but on the other, its association with PTH levels[20,58,59] has been objectified. Thus, in a study of 6,469 people from a general population free of established cardiovascular disease, with a mean follow-up of 8.4 years, after comparing patients with PTH levels <65 pg/ml and PTH ≥65 pg/ml , the latter had a 50% (95% CI: 3-20%) of greater risk of incidence of CI and 5.3 g (95% CI: 2.6-7.9 g) more left ventricular mass determined by RNM[58]. Likewise, in a cohort of 3,713 men between 60-79 years with and without cardiovascular disease, it was observed that in patients with PTH levels >55.6 pg/ml, there was an increased risk of de novo HF (HR=1, 66; 95% CI: 1.30-2.1)[59]. These findings were previously demonstrated by Kestenbaum et al. in a study of 2,312 healthy subjects ≥65 years of age, in which, after a follow-up of 14 years, they observed that patients with PTH ≥65 pg/ml had a greater risk of 30% (95% CI: 6-61%) of incidence of HF[20].

This is interesting given that high levels of PTH generally identify patients with low levels of vitamin D, and the relationship between hypovitaminosis D and PTH levels with HF can be confused. In fact, the progressive deterioration of renal function, physical inactivity, as well as the reduction of calcium absorption, are both causes and consequences of hypovitaminosis D, which in turn

are associated with an increase in PTH levels. Therefore, in the light of the studies previously exposed, it can be extrapolated that it has been shown that there is an independent association of heart failure risk in patients with low vitamin D levels or elevated PTH levels. This is interesting, as some authors consider that it is the levels of PTH that predict cardiovascular disease[60].

Finally, several studies have been published in recent years in which not only a high prevalence of vitamin D deficiency has been observed in patients with HF, but vitamin D has also been linked as a marker of more severe disease and higher rate of adverse events in patients with heart failure. An inverse relationship between 25-HCC levels and B natriuteric peptide (BNP) levels has been observed in patients with HF[20,54], as well as ventricular function[61], reporting as an independent marker of hospitalization due to HF and mortality[20].

However, there is also the theory that vitamin D deficiency in patients with HF occurs because these patients have a worse functional class, are weaker and, therefore, have a more sedentary lifestyle, so they have less exposure to sunlight, which conditions a lower production of vitamin D in the skin and lower concentrations of vitamin D[62]. This is called into question in different studies in which, after a multivariate analysis with the quantification of physical activity, an association between vitamin D levels and ventricular dysfunction and mortality due to HF is still observed[56].

Finally, it is also interesting to comment on the strong association that has been reported among patients with atrial fibrillation and HF (since atrial fibrillation is an important trigger for exacerbation of HF and therapeutic failure) in an observational study in which 180 patients were included. separated into two groups, based on whether they were in sinus rhythm or permanent atrial fibrillation[56]. In the atrial fibrillation group it was observed that plasma levels of vitamin D were significantly lower (11.05 ng/ml *versus* 20 ng/ml; p<0.001), PTH levels were significantly higher (76.7 *versus* 55 pg/ml; p<0.001), and the atrial size was significantly larger (45.03 mm/m^2 *versus* 42.05 mm/m^2; p<0.01) than in the sinus rhythm group. Vitamin D levels (OR=0.854; 95% CI: 0.805-0.907; p<0.001) and atrial size/body surface area (OR=1.077; 95% CI: 1.003-1.156; p were found to be independent predictors of atrial fibrillation <0.05). In this study, the level of vitamin D was established as a predictive cut-off point for atrial fibrillation at 16.50 ng/ml (76.0% sensitivity and 65.5% specificity, area under curve -AUC- =0.75; 95% CI: 0.67-0.82).

In conclusion, there is experimental and clinical evidence that demonstrates plausible pathophysiological mechanisms and a direct and indirect association between vitamin D with HF and the cardiovascular system. Vitamin D deficiency is very high in patients with HF and could be associated with the prognosis of these patients[4,6,8,10,17,19,20,24,30,31,35-37].

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