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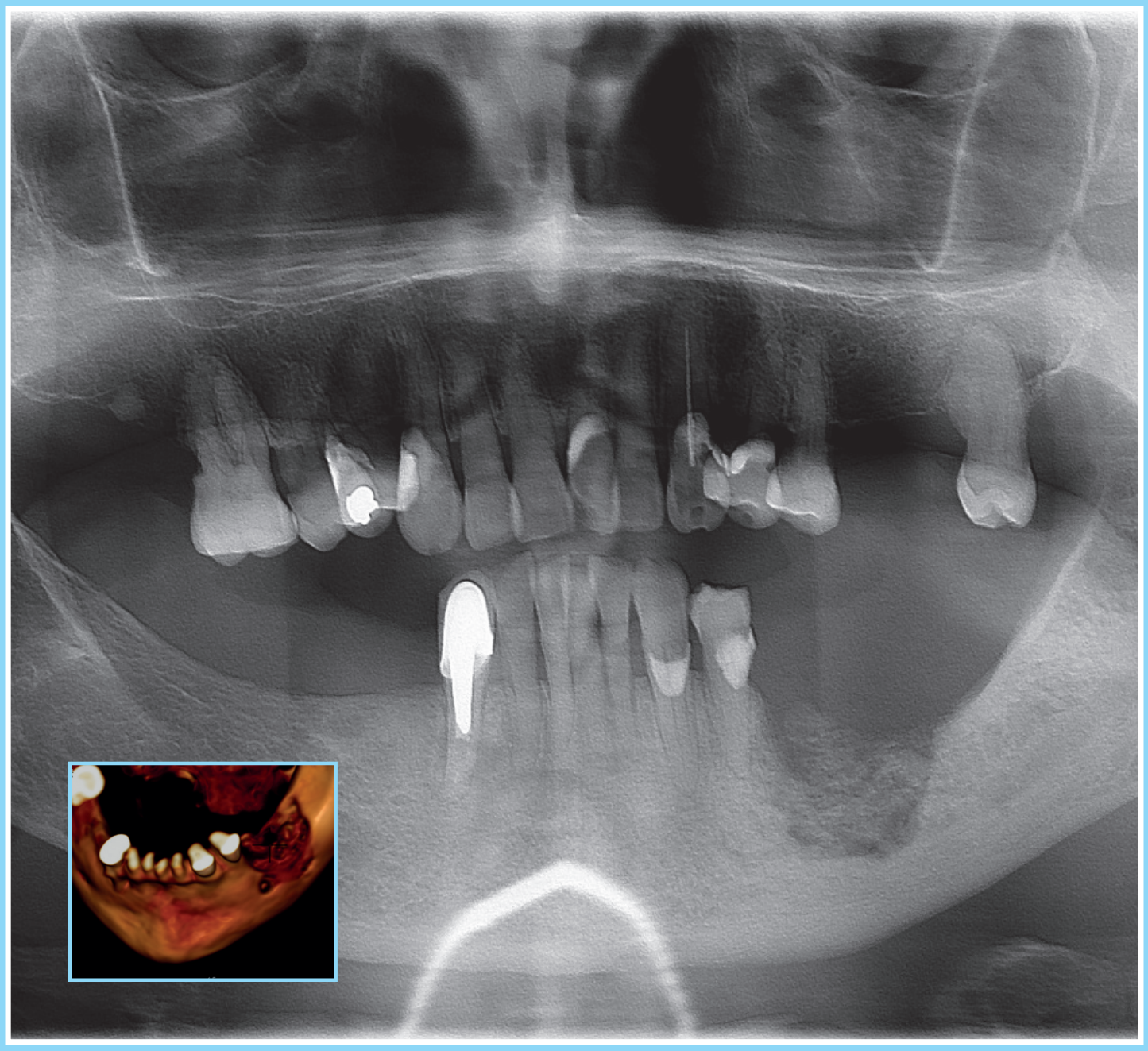

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Summary

Vol. **16** ■ January-March ■ **No.** 1

Originals

Clinical Setting and Decision-Making

Case Reports

Cover images:

Orthopantomography and 3D reconstruction of a patient with mandibular osteonecrosis after extraction of two teeth, under denosumab treatment for 5 years. Bone lysis and presence of bone sequestration.

Courtesy of Drs. Luis M. Junquera and Carlos Gómez from the Maxillofacial Surgery and Bone Metabolism Services at the Hospital Universitario Central de Asturias (Spain).

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Original

PTHrP-stimulated osteocytes stimulated prevent the differentiation of osteoclasts through the modulation of the cytokines CXCL5 and IL-6

Irene Tirado-Cabrera, Joan Pizarro-Gómez, Sara Heredero-Jiménez, Eduardo Martín-Guerrero, Juan A. Ardura, Arancha R. Gortázar

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Abstract

Osteocytes respond to mechanical forces by controlling the function of osteoblasts and osteoclasts. Mechanical stimulation decreases osteocyte apoptosis and promotes bone formation. However, the lack of mechanical load induces osteocytes to favor osteoclastic migration and differentiation, ultimately resulting in bone mass loss. The primary cilium has been described as an important mechanoreceptor in bone cells. PTH1R, the type 1 receptor of parathyroid hormone (PTH), modulates the effects on osteoblasts, osteoclasts, and osteocytes upon activation by PTH or parathyroid hormone-related protein (PTHrP) in osteoblastic cells. Recently, it has been described that mechanical stimulation in osteocytes inhibits osteoclast recruitment and differentiation through a mechanism dependent on PTH1R and the primary cilium. Mechanical stimulation in osteocytes induces the translocation of PTH1R to the primary cilium in MLO-Y4 osteocytes. In this work, we propose to study whether PTHrP reproduces the effects observed with mechanical stimulation regarding the relocation of the receptor to the primary cilium and whether it is also capable of inhibiting osteoclast differentiation through the regulation of cytokines CXCL5 and IL-6. Our results show that stimulation with PTHrP (1-37) triggers a significant mobilization of PTH1R along with the primary cilium in MLO-Y4 osteocytic cells. Additionally, it is observed that PTHrP inhibits osteoclast differentiation through cytokines CXCL5 and IL-6.

Keywords:
Osteocytes. Osteoclasts. PTHrP. CXCL5. IL-6.

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INTRODUCTION

The primary cilium is a unique and immobile appendicular organelle found in numerous cell types, including bone cells, where it functions similarly to an antenna, receiving chemical and mechanical signals (1). Despite being present in almost all human cells, its presence is not constant, as it assembles and resorbs in a cell cycle-dependent process: it forms during the quiescent phase and disappears before entering mitosis (1,2).

Mechanical load is one of the most relevant modulators for the formation and maintenance of bone mass and architecture (3). The presence of the primary cilium in osteocytes is essential for mechanical function (4-6). Besides its role as a mechanoreceptor, the primary cilium accumulates a large number of receptors, including the type 1 receptor for parathyroid hormone (PTH) and the parathyroid hormone-related protein (PTHrP), PTH1R (7-9).

PTH1R is expressed in osteoblasts and osteocytes in which it plays major roles in the regulation of bone metabolism (10). PTHrP is a cytokine expressed in numerous tissues. In bone tissue, it exerts local functions mainly through the PTH1R receptor, although other effects independent of this receptor are known (11-14). PTH1R, in addition to being activated upon binding to its agonists, PTH and PTHrP, is sensitive to mechanical stimuli. It has been reported that mechanical stimulation can directly activate PTH1R in the absence of a ligand, indicating the importance of the PTH receptor as a mechanosensor in osteocytes and osteoblasts (15,16). Therefore, our group has demonstrated that PTH1R is a fundamental component in the process of mechanical signal transduction in MLO-Y4 osteocytic cells (16,17). In these cells, PTH1R activation in the absence of a ligand occurs immediately after mechanical stimulation with fluid flow, thanks to an increase in intracellular calcium influx (18). Moreover, fluid flow induces an increase in PTH1R on the plasma membrane of MLO-Y4 cells (19).

Recently, we have demonstrated that mechanical stimulation by fluid flow in MLO-Y4 cells induces the translocation of PTH1R to the primary cilium (20). Under these conditions, osteocytes inhibit the migration and differentiation of osteoclastic precursors through the alteration of the secretion of the cytokines CXCL5 and IL-6 (21). Although the presence of the PTH1R receptor in the primary cilium is also relevant for the actions of PTHrP (22), it is unknown whether PTHrP (1-37) induces changes in the translocation of the receptor to the primary cilium similarly to those exerted by mechanical stimulation.

In the present work, we aim to study the effects of stimulation with PTHrP (1-37) in the MLO-Y4 osteocyte line on the mobility of the PTH1R receptor in the plasma membrane, analyzing the possible colocalization of the receptor in the primary cilium, as well as the involvement of cytokines CXCL5 and IL-6, produced by osteocytes after stimulation with PTHrP, in the differentiation of osteoclastic precursors.

MATERIALS AND METHODS

CELL CULTURE

Mouse MLO-Y4 osteocytic cells (generously donated by Dr. Lynda Bonewald) were cultured in α-MEM supplemented with 2.5 % fetal calf serum (FCS), 2.5 % fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 µg/mL) in a humidified incubator with 5 % CO_2 at 37 °C. Cells were seeded at a density of 25,000 cells/cm² in conventional culture plates or on glass coverslips (FlexCell International Corp., Hillsborough, NC, United States) coated with a type I collagen matrix (Sigma Aldrich, St. Louis, MO, United States). The cells were maintained in culture until they were almost fully confluent. The next day, the culture medium was replaced with α-MEM without phenol red containing 1 % FBS, and the cells were kept under this condition for 24 hours. Subsequently, the cells were stimulated with the exogenous peptide PTHrP (1-37) (Bachem, Bubendorf, Switzerland) at a concentration of 100 nM for 10 minutes. The unstimulated cells served as static controls (SC). Afterwards, the cells then incubated for 18 hours with α-MEM without phenol red, without FBS or FCS. After this time, conditioned media (CM) was collected. Additionally, blood was obtained from the Blood Transfusion Center of the Community of Madrid. Peripheral blood mononuclear cells (PBMCs) for generating human osteoclasts in vitro were isolated from the buffy coat. These cells were cultured in α-MEM supplemented with 10 % FCS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin, in a humidified atmosphere with 5 % CO_2 at 37 °C. Human monocytes were grown until they reached 90 % confluence.

INHIBITION OF THE PRIMARY CILIUM, PTH1R, GLI1, cAMP, AND PHOSPHOLIPASE C

The formation of the primary cilium and the activity of PTH1R were inhibited by treating the cells for 1 hour with 1 mM aqueous chloral hydrate or 100 nM PTHrP (7-34), respectively. The Gli transcription factor was inhibited using 10 µM Gli-1-Antagonist 61 (GANT61; Santa Cruz Biotechnology) for 1 hour as appropriate. cAMP and phospholipase C were inhibited for 1 hour with 100 µM of the adenylate cyclase inhibitor SQ22536 or with 1 µM of the phospholipase C inhibitor U73122, respectively.

CELL TRANSFECTION

MLO-Y4 cells were transiently transfected with a plasmid carrying complementary DNA (cDNA) encoding human PTH1R fused with the green fluorescent protein (GFP) reporter gene (*^{GFP}PTH1R*) (generously donated by Dr. Peter Friedman) using Lipofectamine 3000 (Life Technologies) for 4 hours at 37 °C, following the manufacturer's guidelines.

IMMUNOFLUORESCENCE

Cells were fixed using 4 % paraformaldehyde (PFA) in PBS, pH 7.4, for 10 minutes and subsequently permeabilized with 0.5 % Triton X-100 in PBS, pH 7.4 for 5 minutes. To block nonspecific binding, the cells were incubated for 1 hour with 10 % bovine serum albumin (BSA) supplemented with 5 % goat serum. Afterwards, they were incubated with the monoclonal antibody produced in mouse anti-α-acetylated tubulin (Sigma Aldrich), diluted 1:1000 in 1X BSA (100 mL of 10 % BSA diluted in 900 mL of PBS), overnight at 4 °C with agitation. The next day, several washes were performed with 1X PBS, and the secondary anti-mouse immunoglobulin G (IgG) antibody conjugated with Alexa Fluor 546 (Invitrogen, Waltham, MA, United States), diluted 1/1000 in 1X BSA, was added for 1 hour at room temperature. Subsequently, several washes were performed with 1X PBS, and the mounting was performed with FluorSafe reagent (Calbiochem, La Jolla, CA, USA). The samples were analyzed with the Leica DMI8 confocal microscope, evaluating the colocalization between the primary cilium and the PTH1R receptor in cells transfected with the *^{GFP}PTH1R* plasmid, while considering whether the colocalization occurred only at the base or along the entire primary cilium. The length of the primary cilium was also analyzed using ImageJ software.

GENERATION OF OSTEOCLASTS FOR OSTEOCLASTOGENESIS EVALUATION

Human monocytes obtained from the isolation of the buffy coat were used for the differentiation assay into osteoclasts. Once they reached 90 % confluence, the culture medium was removed, washed twice with 1X PBS, and trypsinized (in 0.25 % v/v trypsin and 1 mM ethylenediaminetetraacetic acid [EDTA]) for 30 minutes, using a cell scraper. For the differentiation assay, 20,000 cells/well were seeded in a 96-well plate with α-MEM culture medium supplemented with 10 % FCS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin plus 20 ng/mL of M-CSF and 20 ng/mL of RANKL (ProSpec, Ness-Ziona, Israel). The controls were cultured in the same medium but without RANKL. After three days, the culture medium was replaced with fresh medium without phenol red, containing M-CSF and RANKL, as appropriate, and additionally, 20 % of the various CM from MLO-Y4 cells was added. Controls received 20 % α-MEM medium without FCS. Additionally, neutralizing antibodies were added; 2 µg/mL of anti-CXCL5 and 1 µg/mL of anti-IL-6. Human monocytes were in contact with the CM from MLO-Y4 cells for 3 days. After this time, the culture medium was removed and washed with 1X PBS to eliminate non-adherent cells. Cells were fixed with 4 % PFA for 10 minutes, permeabilized with 100 % methanol for 20 minutes, and stained with hematoxylin for 5 minutes. The differentiation of human monocytes into osteoclasts was determined by the morphology of the obtained cells, observing the formation of giant cells with 3 or more nuclei. Images were obtained using the Leica DMI1 microscope, evaluating the number of osteoclastic cells with three or more nuclei using ImageJ software.

STATISTICAL ANALYSIS

Data are expressed as means ± standard deviations (SD). Statistical analysis was performed using GraphPad Prism (GraphPad software). Differences between conditions were evaluated using non-parametric analysis of variance (Kruskal-Wallis) followed by the Mann-Whitney test. A $p < 0.05$ was considered significant.

RESULTS

LOCALIZATION OF THE PTH1R IN THE PRIMARY CILIUM OF PTHrP-STIMULATED OSTEOCYTES (1-37)

First, we aimed to assess how treatment with PTHrP affects the mobility of PTH1R towards the primary cilium in MLO-Y4 osteocytic cells. Confocal microscopy revealed that PTH1R colocalized with the primary cilium in MLO-Y4 cells. Under static conditions, 52 % of ciliated osteocytic cells showed some colocalization between PTH1R and the primary cilium. Of these, only 33 % of the cells showed colocalization along the entire primary cilium, while 19 % showed colocalization only at the base of the cilium. However, after stimulation with PTHrP, we observed a significant increase in the presence of PTH1R along the entire primary cilium (79 % of ciliated osteocytic cells showed colocalization between PTH1R and the primary cilium; of this percentage, 50 % of the ciliated cells showed localization of PTH1R along the entire length of the cilium, while 29 % showed colocalization only at the base of the cilium) (Fig. 1 A and B). Pretreatment with CH inhibited

the formation of the primary cilium as well as the mobilization of PTH1R towards it, while treatment with the PTHrP (7-34) antagonist did not significantly affect the mobilization of PTH1R (Fig. 1 A and B). Under static conditions and inhibition with PTHrP (7-34), 58 % of ciliated osteocytic cells showed some colocalization between PTH1R and the primary cilium. Of these, only 36 % of the cells showed colocalization along the entire primary cilium, while 22 % showed colocalization only at the base of the cilium. After treatment with PTHrP, 40 % of ciliated osteocytic cells showed colocalization between PTH1R and the primary cilium. Of this percentage, 23 % of the ciliated cells showed localization of PTH1R along the entire length of the cilium, while 17 % showed colocalization only at the base of the cilium (Fig. 1 A and B).

We also wanted to determine if the length of the cilium experienced any changes when stimulated with PTHrP (1-37) compared to static conditions. In both cases, the primary cilium had a similar length (Fig. 1C). This effect was inhibited by pretreatment with CH but was not affected by the PTH1R antagonist, PTHrP (7-34) (Fig. 1C).

These results show that stimulation with PTHrP triggers a significant mobilization of the PTH1R receptor to the primary cilium in MLO-Y4 osteocytic cells.

INVOLVEMENT OF CXCL5 AND IL-6 IN OSTEOCLAST DIFFERENTIATION

The regulation of the secretion of CXCL5 and IL-6 cytokines in MLO-Y4 osteocytes through mechanical stimulation has been shown to play a role in the recruitment and differentiation of osteoclastic precursors (21). This regulation associated with mechanotransduction is mediated by the primary cilium and the activation of PTH1R (21). Based on this background and the results obtained regarding the translocation of PTH1R to the cilium (Fig. 1), we wanted to verify whether the cytokines CXCL5 and IL-6 also mediate the actions performed by PTHrP in the communication between osteocytes and osteoclasts using specific neutralizing antibodies (Figs. 2 and 3). The results showed that the conditioned media (CM) from osteocytes under static conditions caused an increase in osteoclast differentiation; an effect that was inhibited by pretreatment of the osteocyte CM with the CXCL5 neutralizing antibody or the IL-6 neutralizing antibody. Additionally, the CM from osteocytes treated with PTHrP (1-37) decreased differentiation towards osteoclasts with or without neutralization of CXCL5 or IL-6 (Figs. 2 and 3). The neutralization of CXCL5 did not cause any effect on osteoclast differentiation when the primary cilium or PTH1R in osteocytes was inhibited with CH or PTHrP (7-34), respectively (Fig. 2 A and B). Similarly, the neutralization of CXCL5 also did not cause any ef-

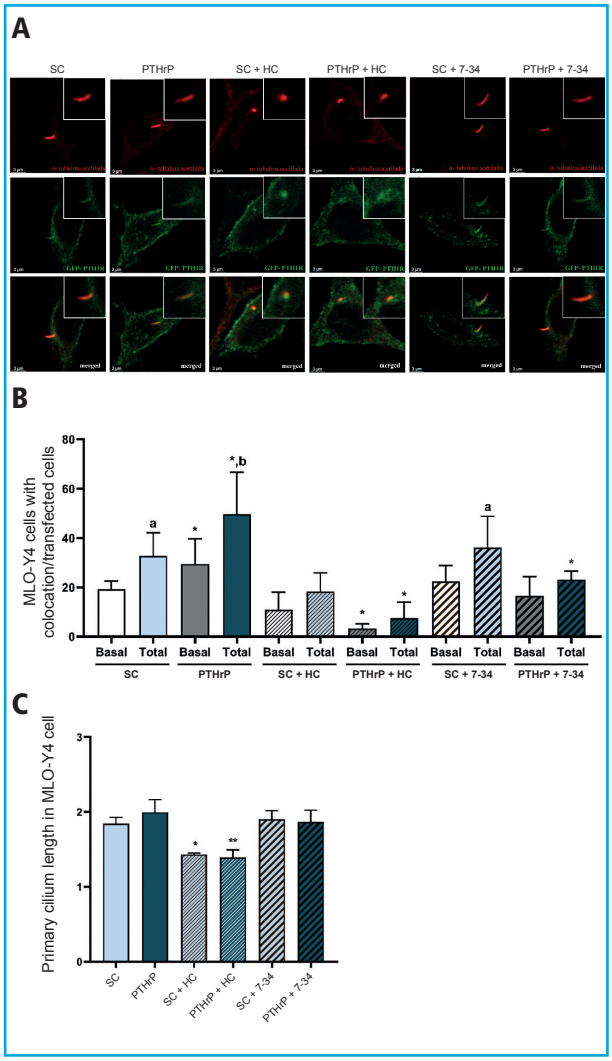

Figure 1. PTH1R colocalizes with the primary cilium in PTHrP-stimulated MLO-Y4 cells (1-37). MLO-Y4 cells were transfected with 1 µg of the $^{GFP}PTH1R$ plasmid using Lipofectamine 3000 for 4 h at 37 °C. Subsequently, the cells were serum-starved for 6 h, treated with 1 mM chloral hydrate or 100 nM PTHrP (7-34) for 1 h, and then stimulated with 100 nM PTHrP (7-34) for 1 h. Afterward, they were stimulated with 100 nM PTHrP (1-37) for 10 min. To evaluate the colocalization of PTH1R with the primary cilium, the cells were fixed, permeabilized, blocked, and incubated overnight at 4 °C with the mouse anti-α-acetylated tubulin antibody. The cells were then incubated for 1 h with the Alexa Fluor 546-conjugated anti-mouse IgG secondary antibody. Representative images of each condition are shown (A). The percentage of cells with PTH1R colocalization at the base (basal) and along the entire primary cilium (total) was analyzed under each condition in MLO-Y4 cells transfected with the $^{GFP}PTH1R$ plasmid (B). The length of the primary cilium in MLO-Y4 cells was evaluated using ImageJ software (C). The results are the mean ± SD of triplicates. $*p < 0.05$ vs. corresponding control; $**p < 0.01$ vs. corresponding control; $^{a}p < 0.05$ vs. corresponding basal condition; $^{b}p < 0.001$ vs. corresponding basal condition (SC, static control; PTHrP, parathyroid hormone-related protein; HC, chloral hydrate; 7-34: PTHrP (7-34); $^{GFP}PTH1R$, green fluorescent protein-PTH1R; PTH1R, type 1 PTH receptor; IgG, immunoglobulin G; SD, standard deviation).

fect on osteoclastogenesis when the GLI, PKA, and PKC pathways were inhibited with GANT61, SQ22536, and U73122, respectively (Fig. 3 A and B). On the contrary, the anti-IL-6 antibody not only reversed osteoclastogenesis under static conditions but also in the presence of CH or PTHrP (7-34) (Fig. 2 A and B). Similarly, inhibition of the GLI, PKA, and PKC pathways also reversed osteoclastogenesis (Fig. 3 A and B).

These findings indicate that both a functional primary cilium and the PTH1R receptor in osteocytes are necessary for proper communication with osteoclasts and suggest that stimulation with PTHrP in osteocytes inhibits osteoclast differentiation through CXCL5, while the activation of PTH1R and the primary cilium in osteocytes regulate osteoclasts through IL-6.

DISCUSSION

The primary cilium is a well-known mechanosensor present in osteocytes among other cells. However, its functions in bone cells do not seem to be limited solely to promoting mechanotransduction. In this work,

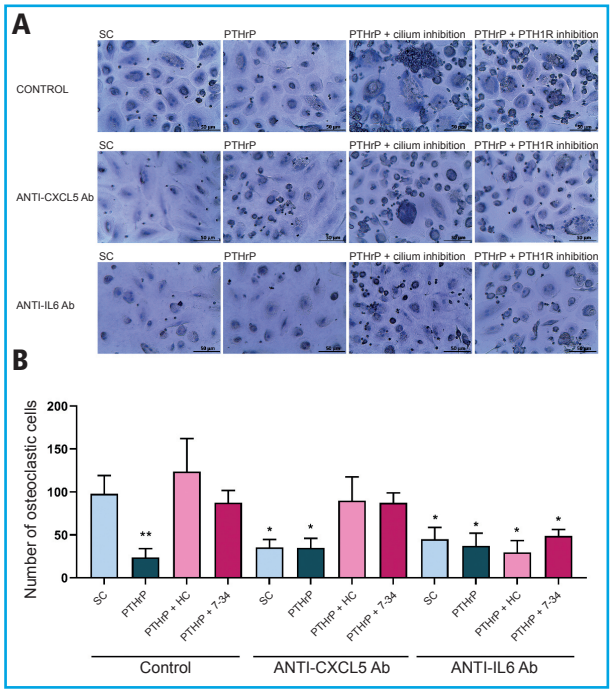

Figure 2. Stimulation with PTHrP (1-37) inhibits osteoclast differentiation through a mechanism dependent on CXCL5 and IL-6. MLO-Y4 osteocytic cells were serum-starved for 24 h and then treated with 1 mM chloral hydrate or 100 nM PTHrP (7-34) for 1 h. The cells were then stimulated with 100 nM PTHrP (1-37) for 10 minutes. CM was collected after 18 h, and 2 µg/mL anti-mCXCL5 neutralizing antibody or 1 µg/mL anti-mIL-6 neutralizing antibody was added. To evaluate the differentiation of monocytes into osteoclasts, human monocytes were treated with 20 ng/mL M-CSF and 20 ng/mL RANKL plus the corresponding CM from MLO-Y4 cells at 20 % with the corresponding neutralizing antibody. The cells were then fixed, permeabilized, and stained with hematoxylin. Representative images of each condition are shown (A). The percentage of cells with three or more nuclei was evaluated using ImageJ software (B). The results represent the mean ± SD of 2 experiments, each in triplicate for each experimental condition. *$p < 0.05$ vs. SC or vs. corresponding inhibition of the cilium or PTH1R; **$p < 0.01$ vs. SC (CM, conditioned medium; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor kappa-B ligand; CXCL5, C-X-C motif chemokine 5; IL-6, interleukin-6; Ab, antibody; SC, static control; PTHrP, parathyroid hormone-related protein; HC, chloral hydrate; 7-34: PTHrP (7-34); SD, standard deviation).

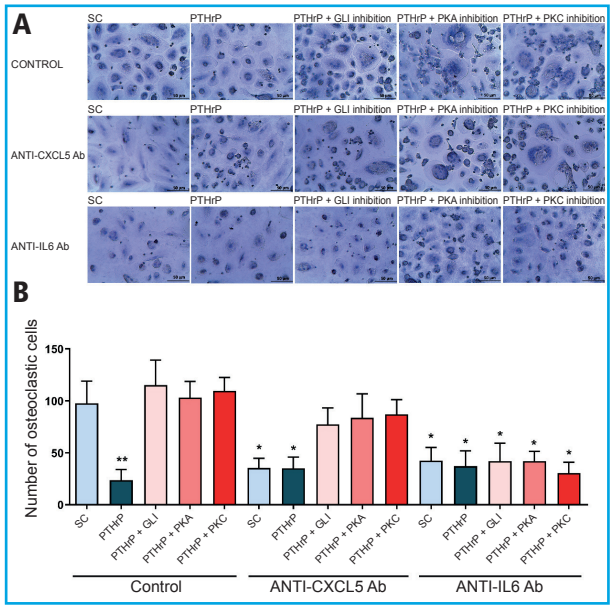

Figure 3. Stimulation with PTHrP (1-37) inhibits osteoclast differentiation through a mechanism dependent on CXCL5 and IL-6. MLO-Y4 osteocytic cells were serum-starved for 24 h and then treated with 10 µM GANT61, 100 µM adenylate cyclase inhibitor SQ22536, or 1 µM phospholipase C inhibitor U73122 for 1 h. The cells were then stimulated with 100 nM PTHrP (1-37) for 10 minutes. CM was collected after 18 h, and 2 µg/mL anti-mCXCL5 neutralizing antibody or 1 µg/mL anti-mIL-6 neutralizing antibody was added. To evaluate the differentiation of monocytes into osteoclasts, human monocytes were treated with 20 ng/mL M-CSF and 20 ng/mL RANKL plus the corresponding CM from MLO-Y4 cells at 20 % with the corresponding neutralizing antibody. The cells were then fixed, permeabilized, and stained with hematoxylin. Representative images of each condition are shown (A). The percentage of cells with three or more nuclei was evaluated using ImageJ software (B). The results represent the mean ± SD of 2 experiments, each in triplicate for each experimental condition. *$p < 0.05$ vs. SC or vs. corresponding inhibition of GLI, PKA, or PKC; **$p < 0.01$ vs. SC (CM, conditioned medium; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor kappa-B ligand; CXCL5, C-X-C motif chemokine 5; IL-6, interleukin-6; Ab, antibody; SC, static control; PTHrP, parathyroid hormone-related protein; GANT61, GLI1-antagonist 61; GLI, glioma-associated oncogene family zinc finger 1 transcription factor; PKA, protein kinase A; PKC, protein kinase C; SD, standard deviation).

our results indicate that the primary cilium exposes the type 1 PTH and PTHrP receptor, PTH1R, along its entire length after stimulation with the PTHrP (1-37) peptide. Additionally, we observed that the cytokines CXCL5 and IL-6 appear to regulate the effects exerted by PTHrP on the communication between osteocytes and osteoclasts, specifically affecting the differentiation of the latter.

PTH1R is expressed in osteoblasts and osteocytes and is key to tissue formation and the maintenance of bone homeostasis (23). PTHrP signaling through PTH1R in osteocytes promotes the inhibition of sclerostin synthesis, leading to increased bone formation, but also activates bone remodeling through the regulation of RANKL (24). PTH1R can be activated by its two agonists, PTH secreted by the parathyroid glands, or PTHrP, secreted by a variety of tissues, such as bone, where it acts locally (10). Several studies indicate that this receptor can act as a mechanoreceptor, being directly activated by mechanical stimuli in the absence of its agonists (25). It has also been described that mechanical load, such as physical exercise, and PTH/PTHrP peptides can synergistically enhance each other's actions (13,26). In fact, physical activity such as running or swimming induces the transient secretion of PTH (27), and mechanical stimulation increases the production of PTHrP in osteocytes, which could induce the activation of PTH1R (18,28).

The primary cilium also acts as a mechanoreceptor in bone cells, receiving mechanical signals. The primary cilium presents numerous receptors and channels capable of being activated by different stimuli. In this work, our results show that after stimulation with the PTHrP (1-37) peptide, there is a migration of PTH1R towards the primary cilium, increasing the presence of the receptor along the entire ciliary projection (Fig. 1). A very similar effect to that described here is observed when these same MLO-Y4 cells are stimulated with mechanical fluid flow (21). Additionally, another study has shown that mechanical stimulation promotes the transport of PTH1R to the primary cilium, increasing PTH signaling in nucleus pulposus cells of the intervertebral disc (9).

Neutralization experiments point to CXCL5 as a cytokine regulated by osteocytes that, when secreted, controls the differentiation of osteoclasts. CXCL5 is a chemokine involved in leukocyte recruitment (29,30). This chemokine binds to the CXC1 receptor and the CXC2 receptor, both expressed in osteoclast precursors (31,32). Additionally, it has been described that CXCL5 modulates the expression of CXC1 and may have a functional role in increasing the expression levels of RANKL in human bone marrow stromal/preosteoblastic cells (33). Sundaram et al. showed that CXCL5 could have a functional role in increasing RANKL expression levels associated with Paget's disease of bone in humans, which presents very localized areas of bone turnover with increased osteoclast activity (33).

Regarding IL-6, its role as an inducer of osteocyte-mediated osteoclastogenesis through the activity of JAK2 and RANKL is well known (34). A study has demonstrated that the increase in IL-6 secretion by apoptotic osteocytic cells promotes the recruitment of osteoclastic precursors. This is because IL-6 secretion promotes endothelial ICAM-1 expression and osteoclastic precursor adhesion (35). The study conducted by Kazuhiro showed that the combination of TNF and IL-6 can induce bone resorption in osteoclast-like cells (36). In fact, IL-6 plays an important role as a regulator during osteoclastogenesis, bone resorption, and regeneration (35). Similarly to this study, osteoclast formation was enhanced after the secretion of both IL-6 and the soluble IL-6 receptor (37). Consistently, our results (Fig. 2 and 3) show that the IL-6 neutralizing antibody decreased cell differentiation when the cilium and PTH1R receptor were inhibited. The same thing occurs when the Hedgehog, adenylate cyclase, and phospholipase C pathways are inhibited. However, the CXCL5 neutralizing antibody had no effect in this regard.

Previous data from our research group show that the silencing of PTH1R is associated with an increase in monocyte migration and osteoclastogenesis, as well as an increase in IL-6 secretion by osteocytes, despite the cells being stimulated by FF. Since IL-6 neutralization under these conditions decreases both migration and differentiation of osteoclasts, these data suggest that the secretion of high levels of IL-6 may overcome the low FF-dependent secretion of CXCL5 and maintain monocyte migration and osteoclastogenesis (20). Similarly, this would occur when stimulation with PTHrP is produced in the process of osteoclastogenesis (Fig. 2).

Moreover, we demonstrated that the inhibition of the primary cilium was also associated with an increase in osteoclastic function, even under conditions of stimulation with PTHrP (1-37), and IL-6 neutralization reversed this effect. Collectively, these data suggest that primary cilium inhibition could induce high IL-6 secretion that overcomes the low CXCL5 secretion, as it occurs when the PTH1R receptor is inhibited (Fig. 4). However, it is also possible that the primary cilium —under conditions of stimulation with PTHrP (1-37)— modulates other alternative cytokines involved in osteoclastic communication. Even so, IL-6 neutralization was sufficient to prevent the differentiation of osteoclastic precursors.

Our findings support that a functional primary cilium and PTH1R are necessary in osteocytes to regulate the secretome of these cells and their communication with osteoclasts. Thus, PTHrP-simulated osteocytes (1-37) inhibit osteoclast differentiation by decreasing CXCL5 secretion, while PTH1R activation and the primary cilium in osteocytes regulate osteoclasts through the modulation of IL-6 secretion.

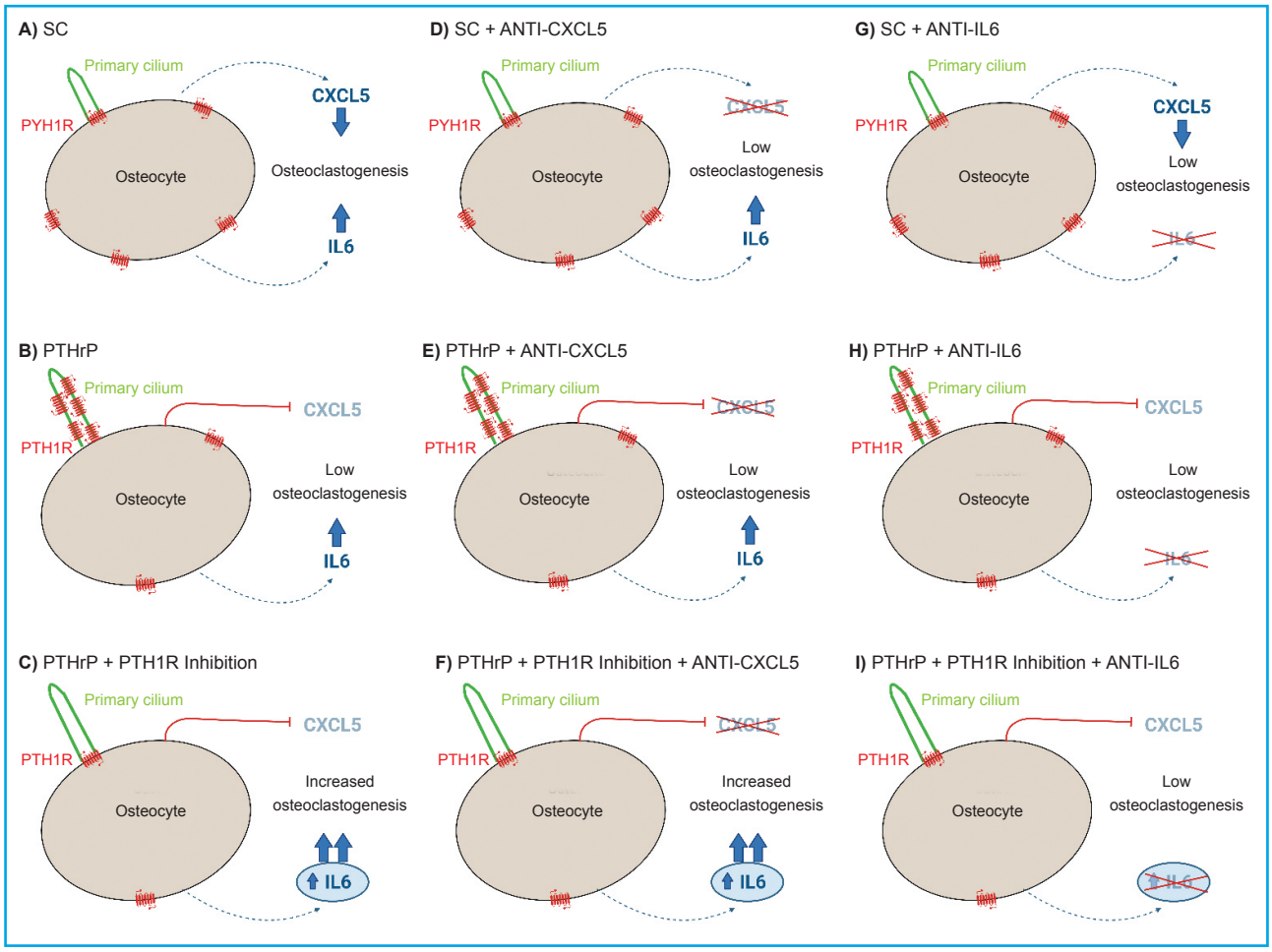

Figure 4. Proposed mechanism for the regulation of osteoclast differentiation by the primary cilium and PTH1R in osteocytes. Both a functional primary cilium and PTH1R in osteocytes are necessary for proper communication with osteoclasts. Stimulation with PTHrP (1-37) inhibits osteoclast differentiation through CXCL5, while PTH1R activation regulates osteoclasts through IL-6 (SC, static control; PTHrP, parathyroid hormone-related protein; CXCL5, C-X-C motif chemokine 5; IL-6, interleukin-6; PTH1R, type I parathyroid hormone receptor).

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Original

Limitations of immunodeficient mice as models for osteoporosis studies

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Abstract

We aimed to establish a murine model to investigate the potential therapeutic role of human mesenchymal stem cells (MSCs) in skeletal disorders *in vivo*. Therefore, we specifically focused on 2 experimental models: the bisphosphonate-related osteonecrosis of the jaw (ONJ) and ovariectomy (OVX)-induced bone loss to simulate postmenopausal osteoporosis. Utilizing NOD.CB17-Prkdcscid/J (NOD-SCID) mice, known for their compromised immune systems, we examined the development of ONJ following varying dosages and routes of administration of zoledronic acid, with and without adjunctive dexamethasone treatment. Surprisingly, we found a very low incidence of ONJ compared to the results reported in immunocompetent mice, suggesting that factors intrinsic to the NOD-SCID mice, such as immune deficiency and possibly altered microbiota due to sterile housing conditions, may influence the development of this condition. On the other hand, these mice did not show the anticipated bone loss following bilateral OVX, challenging conventional wisdom and emphasizing the multifaceted nature of osteoporosis involving both the immune system and microbiota. This study reveals the limitations of immunodeficient mice as experimental models in bone research. On the other hand, it is consistent with experimental data suggesting a role of osteoimmunology and osteomicrobiology mechanisms in the pathogenesis of some skeletal disorders.

Keywords:
Immunodeficient mice. Osteonecrosis. Ovariectomy. Bisphosphonates.

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INTRODUCTION

NOD.CB17-Prkdcscid/J mice, commonly known as NOD-SCID mice, are a genetically engineered strain of mice frequently used in biomedical research (1). These mice are characterized by a severely compromised immune system, due to a specific mutation in the *Prkdc* gene, which encodes a protein kinase vital for DNA repair during immune cell development, and by their background strain, the nonobese diabetic (NOD) mouse, which provides additional unique immune system characteristics. Due to the mutation in the *Prkdc* gene, these mice lack functional T cells and B cells, which makes them unable to mount effective adaptive immune responses (2). This deficiency prevents them from rejecting materials from foreign species, such as human cells. The NOD background contributes to defects in the natural killer (NK) cell function, which is typically less compromised in other SCID strains (2,3). This makes NOD-SCID mice particularly useful for xenograft experiments, in which human cells or tissues are grafted into mice. Therefore, they are extensively used in research involving the transplantation of human cells (1-4).

In the early 2000s, physicians started observing osteonecrosis of the jaw (ONJ) in patients without a history of radiation exposure and realized that most of these patients had breast cancer metastatic to bone or myelomatous disease and were on bisphosphonate (BP) therapy. This led investigators to propose an association between BP use and ONJ (5). The estimated incidence is 1-90 cases per 100 000 patients on BPs per year (6). Most of these cases occur in patients on high doses of BPs (especially zoledronate and pamidronate) due to cancer. Treating ONJ may be rather difficult. In recent years, mesenchymal stem cells (MSCs) have been proposed as candidates for cellular therapies in different conditions including ONJ. MSCs are adult multipotent stromal cells that can differentiate into a variety of cell types, such as osteoblasts, chondrocytes and adipocytes (7,8). Given their ability to differentiate into osteoblasts, they are attractive candidates for bone regeneration therapies. Recent research underscores the multifaceted role of MSCs, mediated not only by differentiation into osteoblasts, but also by the modulation of the bone-healing environment through paracrine effects, involving the secretion of growth factors and cytokines (9-11). Therefore, some studies in animal models have obtained promising results with MSCs applied by either systemic or local routes (12-15). To explore the potentially beneficial effect of a systemic administration of human MSCs *in vivo,* we tried to develop a murine model of ONJ and ovariectomy (OVX)-induced osteoporosis. To prevent rejection, we used immunodeficient mice and protocols previously applied to induce ONJ and osteoporosis in immunocompetent mice.

MATERIAL AND METHODS

NOD.CB17-Prkdcscid/J called NOD-SCID mice were used. Founders were obtained from Jackson Laboratories (Bar, Harbor, Maine, United States) and the colony was housed at the animal facility of University of Cantabria, Santander, Spain under aseptic conditions and veterinary control.

For the ONJ models, 8 week-old mice were anesthetised and the maxillary right first molar was extracted, as published using immunocompetent mice (16). Mice were treated with several intraperitoneal (IP) or intravenous (IV) (retro-orbital plexus) doses of 540 µg/kg of zoledronic acid. The first dose was always given 1 week before the dental extraction, with subsequent doses at weekly intervals. Some mice additionally received subcutaneous dexamethasone (10 mg/kg, 3 times a week). Mice were euthanized 1 week after the last zoledronic acid dose. The maxillary bones were dissected, fixed in formaldehyde and preserved in ethanol prior to study by micro-CT (Bruker). In some experimental mice, as well as in control mice of the same age which did not undergo any experimental procedures, the femur and the tibia were dissected and the trabecular bone volume was determined as previously described (17). After micro-CT analysis, bones were decalcified, paraffin-embedded, and stained with hematoxylin-eosin. Regions of denuded mucosa and bone necrosis (defined as 5 adjacent empty osteocytic lacunae [18]) were evaluated by 2 independent observers.

For the osteoporosis model, female 8-week old NOD-SCID mice were used. Under general anesthesia, both ovaries were exposed and removed. Mice receive opioid analgesia for pain management right after the intervention. A sham-operated group was also included. Eight weeks later, the animals were euthanized, and both the femora and the tibiae were removed and analyzed by micro-CT. T-test was assessed as statistical analysis to compare the differences between mice groups (*p*-values indicated).

Protocol approval was obtained from University of Cantabria Research Ethics Committee and Consejeria de Sanidad de Cantabria, as established by current regulations (code 2016-27930).

RESULTS

OSTEONECROSIS OF THE JAW MODEL

In this study, 27 mice were used to establish an osteonecrosis of the jaw (ONJ) model, receiving varying doses of zoledronic acid administered via IV or IP acces. Table I illustrates the different treatment regimens, including the number of doses, administration route, duration

Table I. Drug treatment and responses in the ONJ group

Treatment	Doses of zoledronic acid (*n*)	Administration route	Time of study (weeks after molar removal)	Mice (*n*)	Osteonecrosis cases (*n*)
Zoledronic acid	5	IP	5	11	0
Zoledronic acid	3	IP	3	3	0
Zoledronic acid, dexamethasone	3	IP	3	2	0
Zoledronic acid	2	IV	2	6	0
Zoledronic acid, dexamethasone	2	IV	2	5	1

IP: intraperitoneal administration group; IV: intravenous administration group.

of the study following molar extraction, and the incidence of osteonecrosis observed among the mice.

Micro-CT analysis confirmed the absence of the first molar, without abnormalities in the maxillary bone (Figs. 1 A and B). Only 1 out of the 27 mice studied showed some evidence of mild ONJ, with a small area of bone with several empty osteocytic lacunae. This mouse had been treated with 2 IV injections of zoledronic acid and dexamethasone (Figs. 1 C and D).

In some representative mice, we measured bone mass to confirm the bioactivity of zoledronic acid. As expected, trabecular bone volume was increased by zoledronic acid via IV or IP access (from a mean 30 ± 8 % to 48 ± 10 % at the femur, and 32 ± 6 % to 48 ± 12 % at the tibia).

OSTEOPOROSIS MODEL

In the evaluation of an osteoporosis model, 24 ovariectomized (OVX) mice and 11 controls (COVX) were successfully included for micro-CT analyses. Unexpectedly, bone mineral density (BMD), bone volume versus tissue volume (BV/TV) parameter, and cortical thickness were similar between the 2 groups (Fig. 2). Specifically, the mean BMD for the femur was 0.20 g/cm² in the OVX group, identical to the COVX group. The BV/TV mean values were 20.3 ± 4.7 % for OVX and 21.8 ± 3.2 for COVX. Hence, no significant differences were found in the bone measured parameters (BMD; p = 0.932; BV/TV p = 0.1507). Dissimilarities were not found either in the femur cortical thickness (p = 0.4242).

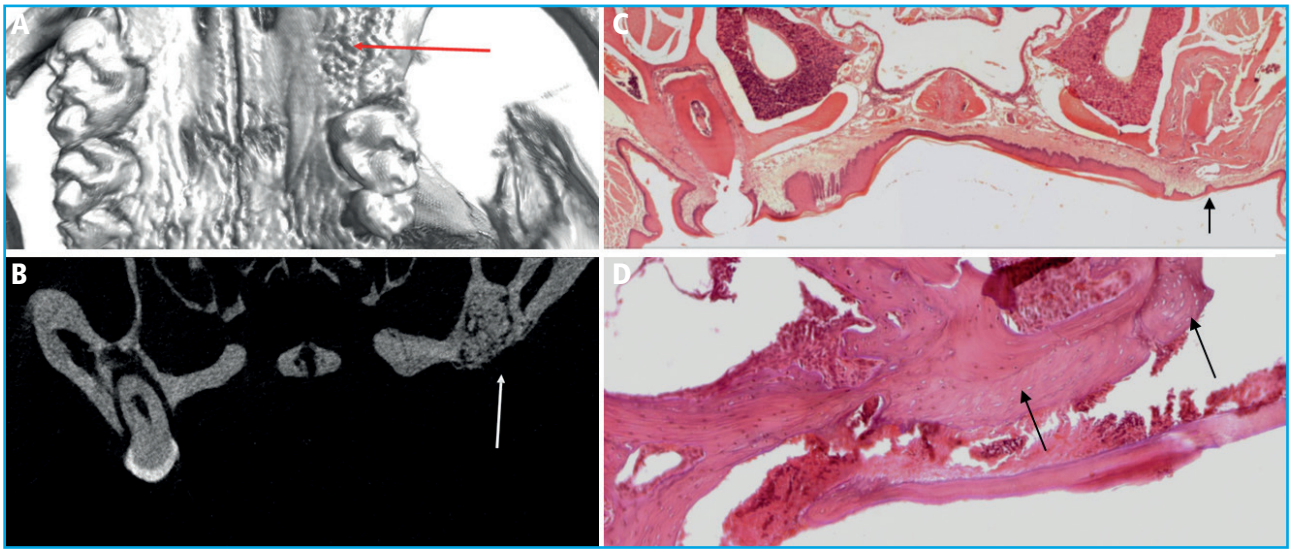

Figure 1. A. 3D reconstruction of the micro-CT analysis showing absence of first molar and cavity filled with new bone (red arrow). B. Coronal image, micro-CT analysis showing the absence of the first molar and the cavity filled with new bone (white arrow). C. Normal healing after tooth removal. HE staining showing complete regeneration of oral mucosa and filling of the cavity (black arrow). Image captured at 4× magnification. D. Mild bone necrosis. HE staining of the only mice showing an area of bone necrosis adjacent to the removed molar cavity (black arrows). Image captured at 10× magnification.

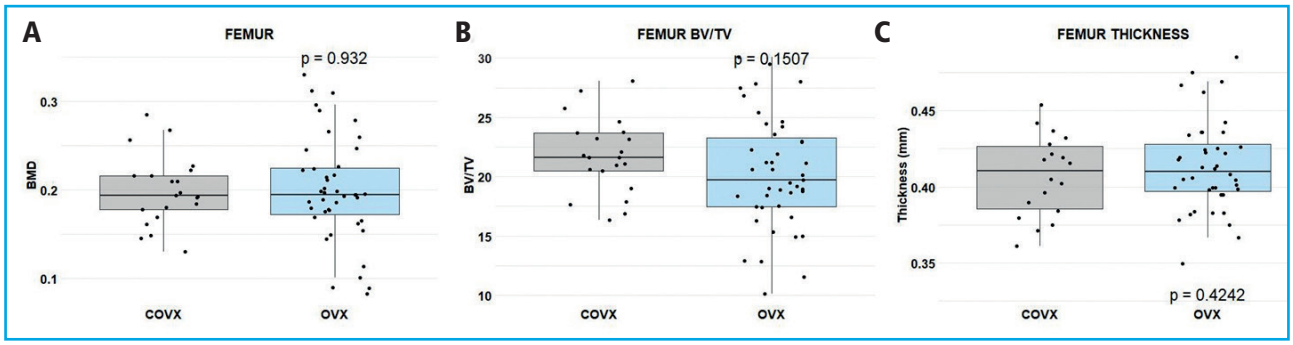

Figure 2. Comparative BMD and cortical thickness analyses with micro-CT. A. Femoral BMD of control (COVX, gray) and ovariectomized (OVX, blue) groups. B. Femoral BV/TV of COVX (gray) vs OVX (blue). C. Femoral cortical thickness of COVX (gray) and OVX (blue) groups. Statistical significance assessed via t-test (*p*-values indicated). Both lower limbs are represented for each mice as an individual point.

DISCUSSION

BPs are the most widely used antiresorptives in the treatment of OP and certain tumors. ONJ is a rare but serious side effect that can arise in patients on antiresorptives. As in other many complex disorders, ONJ seems to be the result of a combination of genetic and environmental factors. There are several well-established acquired risk factors associated with ONJ, such as previous dental extraction, periodontal disease, and the administration of BP (with differences depending on the type and administration route). Local infections and the immune response are also considered part of the ONJ pathogenesis. Different studies have proposed a potential role of Tγδ lymphocytes (18), neutrophils, and macrophages, as well as interleukins and proteins produced by cells involved in the immune system (19).

Despite molar extraction and the injection of high doses of zoledronic acid, only 1 out of the 27 studied mice developed histological findings consistent with mild ONJ. These results widely differ from the 45%-50% of ONJ cases described in studies in immunocompetent mice using a similar methodology (20,21).

Unlike immunocompetent mice, it seems that immunosuppressed mice tend not to develop ONJ even after an insult to the oral mucosa and having received high doses of BP and steroids. Although the mechanisms involved await further studies, we can speculate that it could be due to the immune deficiency *per se* or the sterile conditions in which those mice were maintained, which likely modified oral microbiota. These failed experiments reinforce the concept that factors apart from medication, such as the immune system and the microbiota, play an important role in the occurrence of ONJ after BP therapy.

In line with the ONJ model, the absence of a functional immune system in NOD.CB17-Prkdcscid/J mice, and/or the maintenance of the mice under sterile conditions seemed to prevent them from developing the expected bone loss after OVX. Studies using immunocompetent mice typically report a significant decrease in BMD and changes to bone architecture after 3 weeks following ovariectomy. For example, Smith et al. (2020) observed a significant reduction in BMD and BV/TV and significant increases in trabecular separation in C57BL/6 mice 8 weeks after ovariectomy, highlighting the accelerated bone loss in this model due to normal immune function (22). These differences underscore the critical role of the immune system in post-ovariectomy bone remodeling and the potential for unique disease progression pathways in immunocompromised conditions.

The immune system plays a pivotal role in bone homeostasis, with T cells, B cells, and cytokines influencing both bone resorption and formation. In particular, T cells have been involved in osteoclastogenesis through the production of RANKL, a key osteoclast-activating factor (23). Additionally, classic studies from the Pacifici and Manolagas groups confirmed the role played by cytokines, such as IL-1, IL-6, and tumor necrosis factor, produced by immune and other cells, in OVX-induced osteoporosis (24-26). The lack of an adaptive immune response in these immunodeficient mice could therefore significantly ameliorate the osteoclast-mediated bone resorption expected to occur after ovariectomy.

Moreover, gut microbiota has been increasingly recognized for its influence on bone density and health (27). Microbiota dysbiosis has been associated with inflammatory responses capable of promoting osteoclast activity, eventually leading to bone loss (28-30). The sterile conditions under which NOD.CB17-Prkdcscid/J mice are kept could impact their microbiota, potentially affecting the incidence and progression of bone loss. This concept would be consistent with reports showing that estrogen deficiency causes a gut microbiome-dependent expansion of Th17 cells and TNF-α-producing T cells. This may be of pathophysiological importance, because the blockade of Th17 cell and TNF+ T cell egress from the gut prevented OVX-induced bone loss (31).

In conclusion, the blunted ability to induce BP-related ONJ and OVX-related decrease in bone mass is a limitation for using immunodeficient mice as experimental models in osteoporosis research. On the other hand, they highlight the role of osteoinmmunological mechanisms, and perhaps body microbiota, in bone pathophysiology. The correlation between these mechanisms and their collective impact on bone health requires a broader perspective when investigating osteoporosis and should be taken into account when conducting studies with animal models.

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Revista de Osteoporosis
y Metabolismo Mineral

Clinical Setting and Decision-Making

Osteonecrosis in the context of denosumab — Perspective of the bone metabolism specialist and the maxillofacial specialist

Case report:

A 77-year-old postmenopausal woman, menopausal since age 53, was referred from primary care. She was diagnosed with osteoporosis in another region 6 years ago. The T-score of the densitometry at that time was T-2.8 in the spine, T-3.5 in the femoral neck, and T-3.3 in the total hip. She initially received risedronate, which had to be discontinued due to poor GI tolerance after 1 year on therapy and was then switched to a 6-month regimen of denosumab.

In her personal and family medical history, the patient is hypertensive with good control, while her mother suffered a hip fracture at age 86.

The patient reported that after a dental manipulation, consisting of an extraction and placement of two implants performed 7 months ago, she began experiencing pain and lack of healing in the mandibular area. Her dentist diagnosed osteonecrosis, and since then, she has not had another denosumab injection. After implant removal and local treatment with platelet-rich plasma, her symptoms have improved.

The current densitometry T-score is -2.5 in the spine, -3 in the femoral neck, and -2.8 in the total hip.

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VIEW OF THE BONE METABOLISM SPECIALIST

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The presented case is the archetype of a patient with osteoporosis, with a high risk of fracture, treated with a 6-year regimen of antiresorptive agents, with some efficacy in terms of bone mass and without having experienced fractures during this period. However, she has experienced a rare but characteristic treatment-related complication: osteonecrosis of the jaws (ONJ), although the timing of the dental extraction concerning the denosumab dose is not specified.

A holistic view of the case suggests addressing the local complication, already treated by her dentist, and more importantly, the subsequent clinical management of her osteoporosis.

A global view of the problem may be useful, beyond the particularities of the case presented, to provide a perspective on most cases that may arise.

BONE METABOLIC DISEASE AND DENTAL HEALTH

Dental implications in bone metabolic diseases are a common finding: from the loss of teeth, which is part of the first clinical descriptions of osteoporosis—it is a cardinal manifestation in hypophosphatasia—the association with hypoplastic teeth and the high frequency of caries and destruction of teeth in osteogenesis imperfecta, occlusion alterations in Paget's disease or osteopetrosis, and various complications in primary hyperparathyroidism and renal osteodystrophy (1).

However, what has most transcended in clinical practice in recent years is a very rare complication called osteonecrosis of the jaws (ONJ), associated with antiresorptive treatment in patients with osteoporosis, mainly bisphosphonates (BP) and denosumab. It is important to distinguish from cancer patients who use the same drugs at much higher doses and have a notably higher incidence rate (2).

OSTEONECROSIS AND OSTEONECROSIS OF THE JAWS (ONJ)

It may seem paradoxical that osteonecrosis can appear in any bone, such as bone infarcts or sequestration, asymptomatic when located in central areas of the bone, or having a joint impact if they occur near a joint, as in avascular necrosis of the hip or humeri, but they are not more frequent with antiresorptive treatment and are even used to slow progression (3,4). They are nothing like the antiresorptive-related ONJ.

Possibly the cardinal factor of this difference is that the jaws are located in a septic fossa, separated only by the oral mucosa. Chronic inflammation of the gingival mucosa will cause alveolar bone loss, as in patients with inflammatory arthritis, ONJ weakens the epithelial barrier, and perhaps also alters vascularization (5,6). Hence, additional risk factors for drug-induced ONJ—besides antiresorptives—include diabetes, alcohol consumption, corticosteroid use, immunosuppressants, vascularization inhibitors, smoking, and poor dental hygiene (7).

The first description of ONJ was as an occupational disease in workers with white phosphorus in 1906, such as ceramic decorators who "sharpened" the tips of their brushes with their mouths: phosphonecrosis (formally phosphorus necrosis of the jaw) (8). Subsequently, osteonecrosis associated with local radiotherapy in head/neck tumors is described: radiation osteonecrosis (9). The common denominator of both was the enormous extent of necrosis, abscess formation, and fistulization towards the skin, with severe deformities and very poor prognosis. This consideration is relevant since even today images of these processes are used to teach patients what ONJ can be. In the chapter of historical anecdotes, we should mention that in medical treatment approaches, PENTO therapy was included in these cases—pentoxifylline, tocopherol (to mitigate the vascular component), and clodronate (to improve the bone component) (10), later excluding the bisphosphonate (11).

It was not until 2003 that BP-related ONJ (BRONJ, in international literature) was described, in a patient with multiple myeloma on high doses of pamidronate, with more than 8 weeks of bone exposure in the gingival area (12). Afterwards, the term evolved to antiresorptive-associated osteonecrosis, upon realizing that a potent antiresorptive such as denosumab could also cause the disease, and finally, drug-induced osteonecrosis of the jaws (DIONJ), upon noting an increased risk associated with other drugs unrelated to osteoporosis treatment (13). Of note, the differential diagnosis of the maxillary lesion, beyond concomitant drug use, which includes maxillary sinusitis, deep dental caries, alveolar osteitis, gingivitis and periodontitis, periapical abscess, sarcoma, and chronic sclerosing osteomyelitis (7).

OSTEONECROSIS OF THE JAW ASSOCIATED WITH ANTI-OSTEOPOROTIC DRUGS

The flood of publications on the subject, more than 4700 entries on PubMed, has not been accompanied by a reasonable improvement in the prevention and

treatment of ONJ. The cause is the low incidence rate of this condition, which is also a reason for the discrepancy seen between dental/maxillofacial professionals and osteologists regarding the stochastic component of its appearance (despite recognizing risk factors, perhaps the most relevant being dental interventions). This results in the practical absence of controlled clinical trials on its prevention and/or treatment. As an example, in the excellent critical review by the European Calcified Tissues Society, published in 2022, despite using 254 bibliographic references, the management algorithms often use verbs such as consider, discuss… and very general measures for both osteoporosis and cancer patients, before or during antiresorptive treatment (2).

In our routine clinical practice, we struggle with the most widespread classification of drug-induced ONJ by the American Association of Oral and Maxillofacial Surgeons (2), which, basically includes:

1. "At risk": All asymptomatic patients on drugs with bone effects (reasonable for potent antiresorptives, such as bisphosphonates, denosumab, questionable for romosozumab, yet unacceptable for estrogens, SERMs [raloxifene/bazedoxifene], or even teriparatide).

2. Stage 0: No clinical evidence of necrotic bone, but there are nonspecific symptoms or clinical/radiological findings (any oral symptom in patients on therapy).

3. Stages 1 (exposed bone or fistula without symptoms), 2 (exposed bone or fistula and signs of inflammation), and 3 (with spread beyond the alveolar bone or pathological fracture or extraoral fistulization), which would not be debatable

4. Non-exposed variant (not widely accepted and excluded in the latest updates): unexplained presence of pain in the jaws, fistula, swelling, loose teeth, or mandibular fracture diagnosed after excluding common jaw diseases known to cause similar signs (14).

This classification may be responsible for the different perceptions of incidence between dental professionals and doctors related to bone metabolism.

WHAT IS THE RISK FOR OSTEOPOROTIC PATIENTS AND THE DIFFERENT DRUGS?

There is a notable dispersion in incidence data depending on its source, even with geographical variations, and in the way it is expressed.

For oral BPs, the incidence rate is estimated to be between 1/10,000 and 1/100,000 patient-years of treatment (15), from 0.01 % up to 0.06 % for oral BPs (2), and with an increase after the fourth year of exposure to up to 0.23 % (16), without a clear increased risk for IV BPs (zoledronic 0.9/10,000 patient-years) (13,17), despite some clinical practice guidelines considering it higher risk (18), possibly due to the influence of increased risks seen in cancer indications (19).

In the case of denosumab, the incidence rate in the pivotal study was 5.2/10,000 patient-years, and at 10 years, 35/100,000 patient-years, or 0.30 % (12).

Comparatively, a study attributes a risk of 4.5/10,000 patient-years with oral BPs vs 28.3/10,000 patient-years with denosumab, although two-thirds of the patients from this study had been on oral BP treatment before the addition of denosumab (16,20).

For romosozumab, despite its modest antiresorptive effect, initial data estimate an incidence rate between 0.02 % and 0.03 % (13), derived from the presence of 1 case in the clinical trial vs placebo (21) and 1 case in the sequential treatment group with alendronate (22).

PREVENTIVE MEASURES FOR DRUG-INDUCED ONJ IN OSTEOPOROTIC PATIENTS

It is universally recommended to explore the oral cavity to promote the best possible dental condition before starting potent antiresorptive treatment (2,23,24), although this consideration should include:

- Advising the dental professional about the patient's short-term fracture risk to avoid excessively prolonged dental sanitation, especially in patients with recent fractures and very high fracture risk for whom, for whatever reason, anabolic treatment is not possible.

- Advising the patient about possible dental treatment options, beyond the potential adverse effect of starting antiresorptive treatment. It is not uncommon for theoretically very efficient dental treatments (dental implants) to be suggested for patients who, due to their osteoporosis, do not have sufficient alveolar bone to support the implants and may "lose" them without the use of antiresorptive treatment (Fig. 1), considering alternatives such as bridges, removable prostheses, etc. (Fig. 2).

If the patient is already on antiresorptive treatment, we must advise both the patient and the dental professional that extractions or implants are not contraindicated due to this condition, despite 50 % of dentists believing otherwise (25). The ineffectiveness of determining CTX to predict the risk of osteonecrosis (2,23,24) and, regarding whether to temporarily suspend antiresorptive treatment, the key data are the

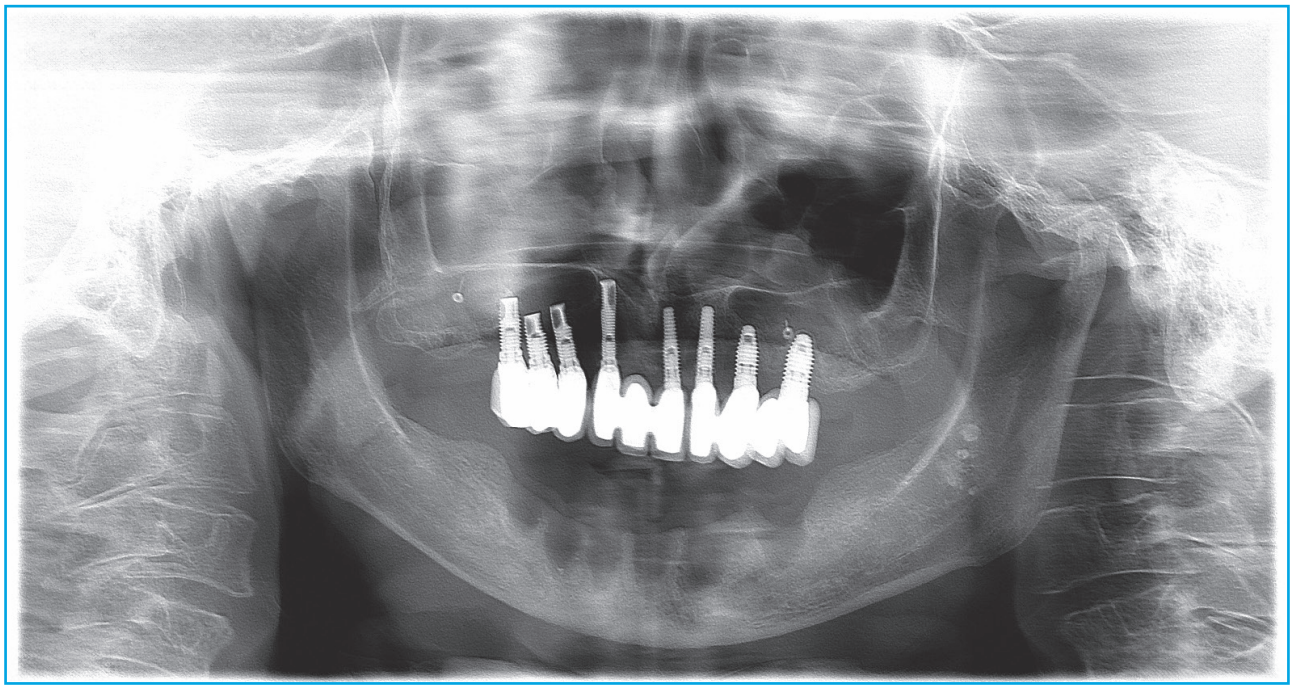

Figure 1. A 69-year-old woman with severe osteoporosis (lumbar spine: -5; femoral neck: -3.2; total hip: -3.6 T), dorsal kyphosis with 2 grade II vertebral fractures. She was referred due to the loss of 6 dental implants in the mandible, and mobility of 2 implants in the maxilla. The patient, untreated for osteoporosis, had been undergoing the implant process for 14 months, including bone grafting to elevate the maxillary sinus. Is this treatment justified?

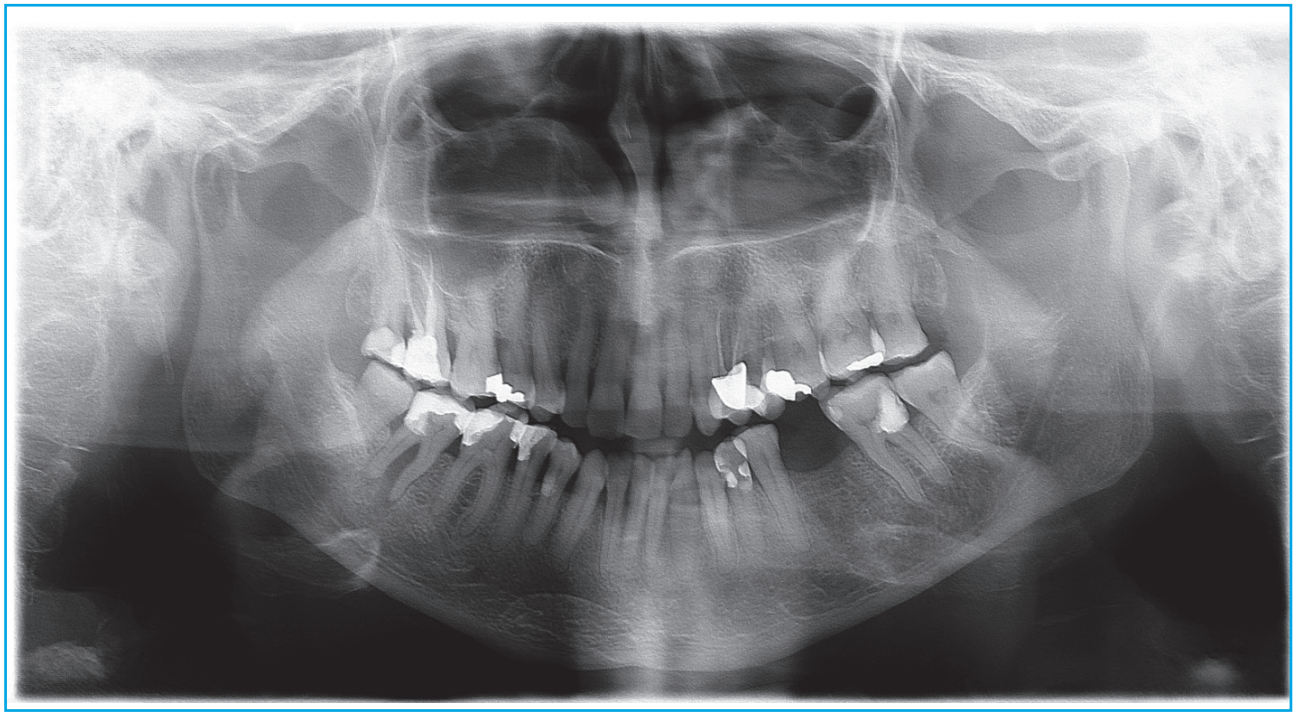

Figure 2. A 66-year-old patient with 3 vertebral fractures, 6 years on denosumab treatment. Baseline BMD of -3.4 T in the lumbar spine and -2.3 T in the femoral neck (currently -2.4 and -1.9 T). Dental extraction without problems. Implant proposed with denosumab discontinuation. What if we opt for a bridge instead?

patient´s fracture risk, as well as the non-association of the risk of ONJ with the qualitative/quantitative state of bone mass (26).

Temporary discontinuation of bisphosphonate treatment has not been shown to be useful in preventing the onset of ONJ (27) due to its pharmacokinetics and persistence in bone tissue. However, strategies, such as suspending oral BP for 4-8 weeks before extraction and reintroducing it once the oral mucosa has healed, may have an anxiolytic effect on the dentist and the patient and does not significantly increase the patient's fracture risk (28,29).

On the opposite pole is denosumab: its suspension entails a significant rebound effect, with an increased risk of fracture, even in the very short term (up to 1 month after the postponed scheduled dose), including multiple vertebral fractures if the patient has prevalent fractures (30,31). In this case, it is advised to perform the intervention (extraction/implant) in the intermediate period between 2 doses28 or at the end of the interdose period (32).

Needless to say, in the case of an acute dental problem that is unresponsive to medical treatment, with clinical persistence and patient suffering, that it is up to the patient to make the decision after receiving truthful information on the personalized risk, even signing an informed consent. It is unacceptable to "wait for a few weeks or months" for the sake of the dentist's peace of mind.

In any case, it is always advisable to take antiseptic measures during the surgical moment, including antibiotic prophylaxis (amoxicillin-clavulanic acid from the day before until completing 8 days in patients who accumulate multiple risk factors) and periodic chlorhexidine rinses, avoid multiple extractions in a single surgical act, and even suture the gingival mucosa after extraction (28,29).

TREATMENT OF ESTABLISHED ONJ

Although the specific treatment of ONJ falls within the competence of the specialist in oral pathology, there are several alternatives and even meta-analyses on the subject (2,28,29,33). However, it is worth mentioning that if considering using teriparatide, absolute or relative contraindications of this treatment must be respected: hypercalcemia, active lithiasis, monoclonal gammopathy of undetermined significance, history of malignant neoplasms, or history of skeletal radiation (24).

In severe cases, surgical management remains the cornerstone of therapy. Antibiotics are the only medical complement with convincing evidence of benefit in DIONJ at present (13).

MANAGEMENT OF OSTEOPOROSIS TREATMENT AFTER ONJ

This is undoubtedly the Gordian knot of the case presented and our routine clinical practice, and as in the above title, the only solution is that of Alexander the Great: cut the knot with a sword.

In terms of morbidity and mortality, the risk of ONJ cannot be compared to the risk posed by osteoporotic fractures. Therefore, patients with osteoporosis and high fracture risk should continue to be treated, even if they have had ONJ.

The key question would be: what is the likelihood of ONJ recurrence in a patient who has already had an episode of ONJ?

There is no relevant literature beyond a few specific case reports (34) on the possibility of ONJ recurrence when antiresorptive treatment persists. Yes, with zoledronic acid in indication and at oncological doses (35).

In the case of denosumab, there are some reports indicating recurrence in neoplastic disease (36), but in the Freedom study and its extension, out of the 11 patients who had ONJ, 8 continued with denosumab—in 7 cases ONJ healed—and no relapse was ever reported (37).

In the case of patients treated with BP, if, as usual, they have been on therapy for 4 years or more, and their incidence of ONJ increases (15) due to the residual effect of accumulated BPs in the bone, we should calm down and space out the reintroduction of the drug (18 months in the case of risedronate and somewhat longer with other BPs [38] due to the residual protective effect against new fractures). Even in patients with very low BMD or coexistence with other notable fracture risk factors (e.g., corticosteroids, multiple fractures, etc.), a cycle of treatment with teriparatide can be interspersed beforehand (24).

In the case of denosumab, discontinuation results in a notable increase in remodeling, loss of bone mass, and an increased risk of fracture, especially if the patient had prevalent vertebral fractures (30,31). In this case, it is known that sequencing treatment with teriparatide is not efficient in the mid-term to contain the increase in remodeling and bone mass loss, with no data on whether this increases the risk of fracture (39). It is known that the transition to an oral BP is not sufficient if the patient has been on denosumab for more than two years, and even in terms of bone mass, the transition to iv zoledronic acid would not be sufficient (with higher theoretical risk of ONJ) (40). The transition to romosozumab due to its modest impact on remodeling and the attenuation of its bone-forming effect (besides some cases of ONJ with romosozumab) does not seem the most efficient al-

ternative either. Therefore, the best treatment to attenuate the rebound effect of denosumab discontinuation would be its reintroduction. There could be an option—in special cases of very high fracture risk or panic crisis after discussing it with the patient—to undergo a period of combined treatment (denosumab-teriparatide) and then, with the patient "stabilized," consider continuing with only denosumab or transitioning to BP.

Exhausting the assumptions, if ONJ occurs in a patient on romosozumab, despite the lack of accurate data on how we should behave, assuming it is likely a patient with very high risk of fracture, it may be continued with the same treatment or, in the absence of contraindications, teriparatide can be used for up to 18-24 months before switching to an antiresorptive.

It goes without saying that if ONJ appears in a patient on teriparatide or SERMs, discontinuation is ill-advised, as there is no data on increased risk of ONJ and, despite its extreme rarity, there are cases of ONJ occurring without any treatment (41).

In conclusion, in the case at hand, the best treatment option would be to continue denosumab treatment, in addition to the local treatment of ONJ.

VIEW OF THE MAXILLOFACIAL SPECIALIST

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Drug-induced osteonecrosis of the jaw (DIONJ) is a rare condition that primarily occurs in patients on IV bisphosphonates.

However, in a hospital like ours (Hospital Universitario La Paz), we see patients who present with osteonecrosis after prolonged treatment with oral bisphosphonates or denosumab. Typically, the development of symptomatic disease is accompanied by surgical manipulation involving the jawbone, especially when it results in bone exposure to the oral cavity, as it is the case after extractions. The case presented here is highly representative of this situation. On the one hand, an extraction was performed, and 2 dental implants were placed. As I mentioned, extraction exposes the bone to an oral cavity populated with potentially pathogenic microorganisms, and the placement of implants requires well-vascularized bone for the process of osseointegration to occur. Thus, we encounter the two situations implicated in the pathogenesis of osteonecrosis: infection and vascularization changes, which in this case have resulted in mandibular osteonecrosis. Fortunately, the process seems to have been limited and affected only the bone in the implant area, leading to bone sequestration that was removed along with the non-integrated implants. At this point, the most important step is to thoroughly debride the underlying bone and ensure good soft tissue coverage. The use of platelet-rich plasma or growth factors can aid in healing.

In our experience, treatment with denosumab does not contraindicate the placement of dental implants. We usually prefer to first perform the extraction, ensure good mucosal coverage of the socket, and delay the placement of implants for about 10-12 weeks after the extraction. We always use preoperative antibiotic prophylaxis and postoperative antibiotic treatment if deemed necessary. Regarding the optimal timing for the procedure, considering that denosumab is administered semiannually, we recommend performing the procedures 4 or 5 months after the administration of the drug.

Finally, on the continuation of pharmacological treatment, one must weigh the benefits of the drug vs the risk of osteonecrosis. If the process has been localized with bone sequestration that has been removed and the bone has healed well, without evidence of disease progression, we believe that treatment could be continued.

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Case Report

New forms of resistance to the action of human parathyroid hormone analogues

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Abstract

Introduction: we report the emergence of resistance to the action of teriparatide in a patient with postsurgical hypoparathyroidism under replacement therapy with this molecule, who initially showed an optimal response.

Case report: description of a case report in which a patient with postsurgical hypoparathyroidism treated with teriparatide showed progressive decrease in its effectiveness, and resistance to the biosimilar teriparatide with which the patient was treated was tested. The Ellsworth-Howard test was used to evaluate teriparatide resistance.

Discussion: the patient showed a response to the Ellsworth-Howard test consistent with resistance to teriparatide. A comparison was made with a patient with chronic hypoparathyroidism who underwent the same test with the same molecule, obtaining an appropriate functional response. The occurrence of primary failure to teriparatide replacement therapy in the context of chronic hypoparathyroidism is presented. Autoimmune etiology due to the development of blocking autoantibodies is the most likely hypothesis.

Keywords:
Teriparatide.
Postsurgical chronic hypoparathyroidism.
Resistance.

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CASE REPORT

We present the case of a 28-year-old male with a history of total thyroidectomy for stage II papillary thyroid carcinoma at the age of 17 years. Following surgery, he developed difficult-to-control hypoparathyroidism, for which he was initiated on replacement therapy with teriparatide 20 mcg subcutaneously every 12 hours in February 2022, resulting in normalization of phosphocalcic metabolism that remained stable for 18 months from the start of treatment (1). Since August 2021, he again presented analytical alterations compatible with hypoparathyroidism, with partial corrections occasionally requiring re-association of calcitriol and calcium carbonate or adjustment of the teriparatide dose to 0.8 mcg/kg/day in 3 doses (20-20-20 mcg subcutaneously) following the protocol described by K.K. Winer, in which the teriparatide dose to achieve normocalcemia without the need for calcitriol or calcium salts ranged from 0.47 + 0.33 mcg/kg/day (2). Analytical evolutions and therapeutic changes are presented in figure 1. Given the doses achieved under replacement therapy with teriparatide (equal to the maximum doses presented in the known case series to date), we considered the possibility of primary treatment failure once therapeutic adherence was ruled out, given the resistance pattern to it.

DISCUSSION

We know that PTH is an 84-amino acid peptide hormone produced in the parathyroid glands. Its amino terminal end 1-34 constitutes the active fraction. Its membrane receptor PTH-PTHrp is coupled to a stimulatory G protein of adenylate cyclase that generates cAMP as a second messenger. At the renal level, it inhibits the expression of Na-P 2A and 2C cotransporters, inducing a phosphaturic response. Both responses (phosphaturic and increased urinary cAMP excretion) have been the basis of PTH functionality studies. In the 1970s, Broadus (3) further delineated the renal

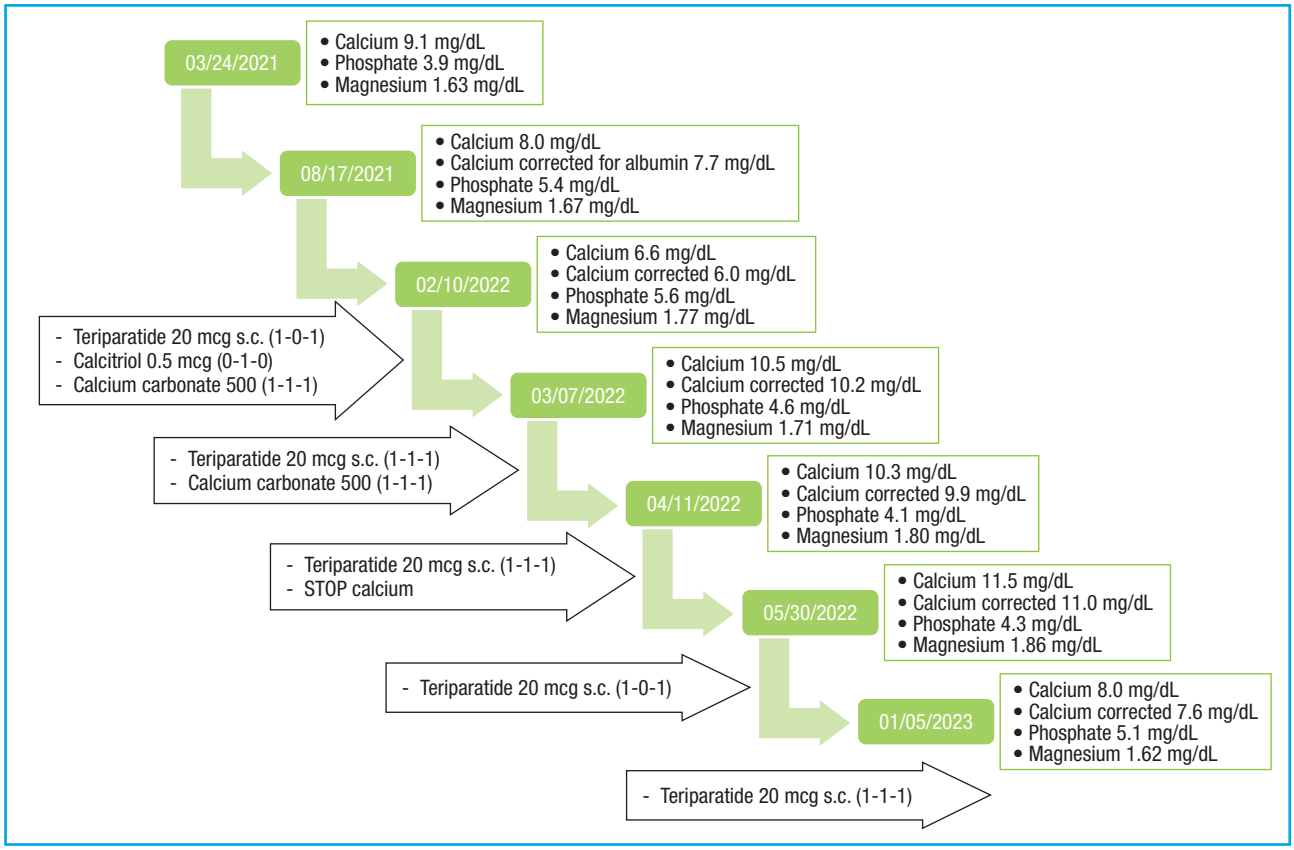

Figure 1. Evolution of phosphocalcic metabolism since August 2021.

response to PTH by determining nephrogenic cAMP (NcAMP) by eliminating factors that could interfere with the interpretation of urinary cAMP excretion (other hormonal hyperfunctions or pharmacological responses mediated by cAMP). The genesis of NcAMP is almost entirely secondary to the renal action of PTH, with a component due to vasopressin practically negligible at physiological levels. These findings led to the standardization of the Ellsworth-Howard test (4) for determining the organic response to PTH. Subsequently, its standardization with teriparatide instead of using purified PTH extract appeared (5). An increase in phosphaturic response of at least 200 % and in nephrogenic cAMP response of at least 1000 % (6) is considered normal; this response is more striking in patients with hypoparathyroidism. Conversely, a lower response is described in states of PTH resistance (pseudohypoparathyroidism).

An Ellsworth-Howard (E-H) test was performed on the case patient (patient 1) administering 20 mcg of teriparatide intravenously by slow infusion over 15 minutes; simultaneously, the same test was performed on a control patient (patient 2) similarly affected by postsurgical hypoparathyroidism and osteoporosis, who was prescribed daily teriparatide 20 mcg for treatment. Both tests followed the same protocol, and the same biomolecule was administered (teriparatide Movymia [STADA Laboratories]). The phosphocalcic metabolism responses of both patients are summarized in the following table (Table I).

Analyzing the response to PTH 1-34 stimulus of patient 1, we observed a blocked phosphaturic response, as well as a suboptimal increase in nephrogenic cAMP. Patient 2, however, presented an optimal phosphaturic response, as well as a significant increase in nephrogenic cAMP genesis (Fig. 2).

In the case of patient 1, suffering from chronic hypoparathyroidism, with an initial optimal response to teriparatide, the decrease in treatment effectiveness and the response to the Ellsworth-Howard test is compatible with primary failure to teriparatide replacement therapy with a pattern of resistance to its action. This conclusion is supported by the adequate response of a control patient (patient 2) with the same underlying condition (chronic hypoparathyroidism under replacement therapy with PTH 1-34) to the E-H test with a teriparatide dose from the same batch and pen as the case reported, ruling out the possibility of adulterated or inactive drug.

In the clinical development of teriparatide Movymia, the presence of anti-teriparatide antibodies was described in 2 out of 126 individuals included in the Forsteo teriparatide group, none of them blocking, and no development of autoantibodies was described in the group assigned to teriparatide Movymia (7). It should be noted that the clinical development studies of teriparatide Movymia were conducted in patients with osteoporosis without hypoparathyroidism, so we cannot extrapolate the results of autoimmunity to our patient.

Table I. Responses to phosphocalcic metabolism

	Patient	0´	15´	30´	60´	120´	360´	Levels of normality
Glomerular filtration	1	> 90	> 90	> 90	> 90	> 90	> 90	> 60 mL/min/1.73 m^2
	2	72	75	75	82	85	78	
PTH	1	< 10						15-65 pg/mL
	2	11.4						
Calcium adjusted to albumin	1	6.9	7.2	7.2	8.08	7.8	6.9	8.4-10.2 md/dL
	2	8.5	8.5	8.54	8.6	8.9	9.2	
Magnesium	1	1.62	1.66	1.65	1.59	1.7	1.53	1.6-2.6 mg/dL
	2	1.81	2.04	1.85	1.82	1.84	1.91	
Phosphate	1	4.6	4.4	4.6	4.8	4.7	4.7	2.5-4.5 mg/dL
	2	4.8	4.4	4.4	4.4	4.5	4.7	
25(OH)-D (25-hydroxyvitamin D)	1	13.5						20-40 mg/mL
	2	20.9						
1.25(OH)-2D (1.25-dihydroxyvitamin D)	1	44						20-54 mg/mL
	2	22						
Fosfaturia	1	329	295	292	269	211	No calc	40-136 mg/dL
	2	772	882	1885	1518	2952	909	
NcAMP	1	0.71	4.76	4.73	1.32	< 0.3	< 0.3	0.30-3.80 nmol/dL FG
	2	> 0.3	119.62	29.75	2.16	> 0.3	< 0.3	

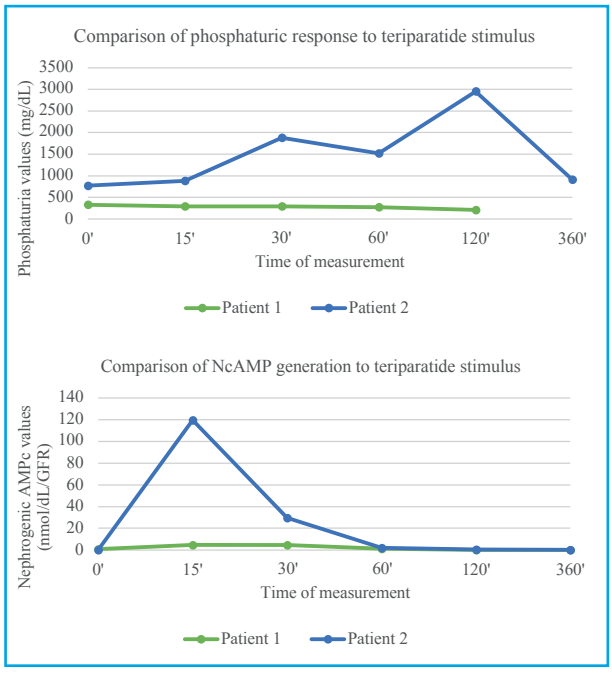

Figure 2. Comparison of Ellsworth-Howard Test results.

To clarify the nature of the blocking substance, attempts were made to request a kit for the determination of anti-teriparatide antibodies (not commercially available) or serial determinations of PTH 1-34 in diluted solutions or precipitation in polyethylene glycol to eliminate the interference of blocking antibodies. However, current third-generation commercial assays for PTH determination have a double binding to fragments 1-38 and 38-84, so these tests could not be performed. Nevertheless, the development of autoantibodies is the main hypothesis for the genesis of the blockade to the action of teriparatide, following the models of primary failure to respond to treatments with other molecules such as biological drugs.

In conclusion, we present our first case of primary failure to teriparatide replacement therapy in a male with long-standing postsurgical chronic hypoparathyroidism, with a response compatible with resistance likely induced by blocking autoantibodies against teriparatide. It would be of interest to study the loss of effectiveness of replacement therapy in patients with these characteristics in the long term and clarify its origin, considering the determination of anti-teriparatide antibodies in case of loss of activity.

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Case Report

Calcium-alkali syndrome: A rare cause of hypercalcemia?

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Abstract

Severe hypercalcemia is an electrolyte disorder with variable clinical expression that can be serious if appropriate corrective measures are not taken. Among the main causes of hypercalcemia are primary hyperparathyroidism and neoplasms. However, it appears that cases of a third cause of secondary hypercalcemia are increasing. The calcium and alkali syndrome, formerly known as milk-alkali syndrome, is a disorder resulting from excessive intake of calcium and alkaline compounds, leading to hypercalcemia, metabolic alkalosis, and renal failure. One of the contributors to this syndrome is the ingestion of calcium carbonate antacids for dyspepsia, which are also used (sometimes along with vitamin D metabolites) to treat bone pathologies or in post-surgical medical treatments following thyroid and parathyroid gland interventions.

We describe four cases of patients who developed this syndrome with severe hipercalcemia in the context of post-surgical hypoparathyroidism treatment, aiming to raise awareness among clinicians and patients about the potential risks of calcium supplements associated with calcitriol.

Keywords:
Hypercalcemia. Alkalosis. Calcium. Carbonate. Calcitriol.

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INTRODUCTION

Hypercalcemia is a relatively common finding in the routine clinical practice. It results from failed renal calcium excretion to compensate for the increased entry of calcium into the circulation (1). Primary hyperparathyroidism and hypercalcemia due to a malignant neoplastic process are among the main causes, although multiple situations can cause elevated serum calcium levels, albeit less frequently (2). Correctly identifying the cause of elevated calcium levels is crucial for applying the appropriate treatment (3).

Calcitriol treatments are indicated for patients with renal osteodystrophy, idiopathic postoperative hypoparathyroidism (4), pseudohypoparathyroidism, vitamin D-dependent rickets (5) or resistant rickets, osteomalacia, and for preoperative treatment in primary hyperparathyroidism. Calcium carbonate is indicated to prevent calcium deficiency and as an adjunct in the management of osteoporosis (6). Both drugs have hypercalcemia as a very common adverse reaction (more than 1 case per 10 patients treated) for calcitriol and less common (more than 1 case per 1000 patients treated) for calcium carbonate.

The calcium-alkali syndrome (CAS) is the most updated term for the widely known milk-alkali syndrome. This condition is characterized by the triad of hypercalcemia, metabolic alkalosis, and varying degrees of renal failure due to calcium ingestion along with an absorbable alkali (7,8).

The following reports 4 cases of CAS that presented with severe hypercalcemia to raise awareness among clinicians and patients on the potential dangers of calcitriol-associated calcium supplements.

CASE REPORTS

CASE REPORT #1

A 77-year-old woman with a history of dyslipidemia and chronic ischemic heart disease, with preserved renal function (serum creatinine levels of 1.15 mg/dL) on enalapril, torasemide, and acetylsalicylic acid. She was diagnosed with primary hyperparathyroidism, for which a right parathyroidectomy was performed. Due to persistent symptoms, another surgery involving left parathyroidectomy and hemithyroidectomy was required. Pharmacological treatment with calcium carbonate (2 g every 24 hours) and calcitriol (0.5 µg every 24 hours) was initiated. Despite treatment, the patient exhibited hypocalcemia with hyperreflexia and a positive Trousseau sign, requiring hospitalization for electrolyte replenishment. The electrocardiogram performed at admission was normal. After discharge, the calcium carbonate dose was increased up to 5 g every 6 hours. In a routine check-up, the patient had calcium levels of 17.1 mg/dL (8.7-10.4 mg/dL) with ionic calcium levels of 2.3 mmol/L (1-1.4 mmol/L), creatinine levels of 1.93 mg/dL (0.5-1.1 mg/dL), bicarbonate levels of 33 mmol/L (22-28 mmol/L), and phosphate levels of 2.2 mg/dL (2.4-5.1 mg/dL), suppressed PTH (15-65 pg/mL), and 25-hydroxyvitamin D (25OHD) of 21.1 ng/mL (30-40 ng/mL). In light of these findings, the attending physician was contacted to expand the lab tests, obtaining a pH value of 7.43 (7.32-7.42).

The patient was called to the hospital, and treatment with calcium carbonate and calcitriol was immediately discontinued. Upon examination, the patient was asymptomatic, with no electrocardiographic signs suggestive of complications from hypercalcemia, so no other corrective measures were applied. In subsequent controls, after the withdrawal of calcitriol and calcium carbonate, the patient showed a progressively improved renal function and a decrease in calcium levels to reference values.

CASE REPORT #2

A 51-year-old man without relevant clinical history or notable analytical changes in previous controls, with recent creatinine levels of 1.14 mg/dL, required total thyroidectomy for retrosternal goiter. After a second surgery due to persistent symptoms, pharmacological treatment with levothyroxine (125 µg every 24 h), calcium carbonate (4.5 g every 24 h), and calcitriol (0.50 µg every 24 hours) was initiated. In a routine check-up, the patient had calcium levels of 13.1 mg/dL with ionic calcium levels of 1.57 mmol/L, creatinine levels of 2.23 mg/dL, a pH of 7.39, bicarbonate levels of 31.1 mmol/L, suppressed PTH, and 25OHD of 27 ng/mL.

The patient was called to the hospital, where the lab test results were confirmed. Upon examination, he presented with back pain, paresthesia, asthenia, and generalized muscle pain that had increased in recent days. The pharmacological treatment was immediately discontinued, and the patient was hospitalized for fluid therapy (300 mL of 0.9% NaCl every 24 h) to hydrate and minimize renal damage, and zoledronic acid was used as an inhibitor of bone resorption to prevent the release of calcium from the bone (a 15-minute 4 mg/5 mL ampoule infusion)9. When calcium levels went back to normal (8.2 mg/dL), the patient was discharged after 4 days of hospitalization. An interconsultation with the Nephrology Unit was conducted due to persistent renal failure, with creatinine values of 1.63 mg/dL. A renal ultrasound detected radiopaque calculi suggestive of calcium oxalate in both kidneys. After getting rid of the stones, the patient showed complete recovery of renal function.

CASE REPORT #3

A 58-year-old woman diagnosed with primary hyperparathyroidism underwent a left parathyroidectomy. Due to the progression of osteoporosis and persistent symptoms, total parathyroidectomy was performed. She was prescribed calcium carbonate (3 g every 24 hours), calcitriol (1 µg every 24 hours), levothyroxine (50 µg every 24 hours), and esomeprazole (40 mg every 24 hours) after surgery. Pre-episode analyses did not show any significant findings. The most recent creatinine determination before the episode was 1.37 mg/dL.

The patient presented to the ER with a several day-history of general malaise, constipation, and reduced urine output, along with nausea, vomiting, and generalized weakness. Lab test results showed creatinine levels of 2.73 mg/dL, calcium levels of 17.3 mg/dL with ionic calcium levels of 2.08 mmol/L, suppressed PTH, pH of 7.40, bicarbonate levels of 33.1 mmol/L, and TSH levels of 1.05 µg/mL (0.27-5.0 µg/mL).

Given these results, the patient was admitted with a diagnosis of renal failure and hypercalcemia. IV fluid therapy (300 mL of 0.9% NaCl every 24 h) was administered to preserve renal function and reduce calcium levels, along with methylprednisolone (40 mg every 12 hours in intermittent dilution) followed by the immediate withdrawal of calcium carbonate and calcitriol. Three days after admission, calcium levels went back to normal (9.8 mg/dL), and creatinine improved slightly (2.17 mg/dL). The patient was discharged, with follow-up in Primary Care.

CASE REPORT #4

A 63-year-old woman with dyslipidemia and depression treated with atorvastatin and fluoxetine had undergone a total thyroidectomy years earlier treated for postoperative hypothyroidism with levothyroxine (100 µg every 24 hours), calcium carbonate (2 g every 12 hours), and calcitriol (0.5 µg every 12 hours). She was admitted to the ER with symptoms of dizziness, weakness, and vomiting, along with a blood pressure of 164/90 mmHg upon initial examination. Previous tests showed no significant changes, and renal function and calcium levels were within the normal range a few months earlier during a routine analysis (creatinine levels of 0.9 mg/dL and total calcium levels of 10.4 mg/dL).

Lab test results showed total calcium levels of 19 mg/dL, ionic calcium levels of 2.08 mmol/L, bicarbonate levels of 31.8 mmol/L, a pH of 7.45, suppressed PTH, and creatinine levels of 1.31 mg/dL, indicating a diagnosis of severe secondary hypercalcemia. Treatment with fluids, methylprednisolone (40 mg every 24 hours in intermittent dilution), and IV zoledronic acid (4 mg/100 mL over 15 minutes) was initiated.

The patient was hospitalized, and calcium carbonate and calcitriol were immediately discontinued. Calcium levels were monitored, reaching values of up to 11.4 mg/dL and a pH of 7.52 after 2 days of hospitalization. The patient remained hospitalized, and on the 4th day, calcium levels were 10.0 mg/dL and pH, 7.42. At discharge, follow-up tests were scheduled 2 months later, and therapy with calcium carbonate and calcitriol was reinstated at reduced doses (500 mg every 12 hours and 0.5 µg per day, respectively).

DISCUSSION

Since the introduction of proton pump inhibitors for the management of dyspepsia, CAS has been considered a rare cause of hypercalcemia (< 1% of cases) (10). However, numerous authors suggest that the incidence of this syndrome is increasing. In recent series studied, it is responsible for more than 12% of hypercalcemia cases. These data position CAS as the third leading cause of hypercalcemia (7) and the second leading cause of severe hypercalcemia (7,8). This situation can be explained by the increased prescription of calcium supplements considered safe for various conditions, such as hypoparathyroidism, or physiological processes like menopause (8-11). However, the cases we present highlight that these drugs can lead to severe hypercalcemia when consumed over prolonged periods.

All our patients had previously undergone surgery, resulting in a status of postoperative hypoparathyroidism with suppressed PTH levels. They were on calcium carbonate, which, in addition to providing calcium that contributes to hypercalcemia, incorporates the alkaline component which is essential for the development of the syndrome. This is because the components of calcium carbonate contribute to the increase of plasma bicarbonate, which decreases its renal excretion (12). None of the patients were previously on ant other treatments that could cause hypercalcemia, such as thiazide diuretics, so the only plausible iatrogenic cause is due to calcitriol and calcium carbonate. Metabolic alkalosis would also increase calcium reabsorption at tubular level. Additionally, the fact that they were on supplements of vitamin D metabolites (calcitriol) with effects on bone resorption, absorption, and reabsorption of calcium at intestinal and renal levels, respectively, and PTH secretion (13), caused a synergistic hypercalcemic effect, contributing to the overall clinical picture (14,15). The technical sheet for calcitriol recommends monitoring calcium levels, at least, twice a week when initiating treatment and, once the optimal dose is adjusted, calcium levels should be checked monthly.

The emergency treatment of CAS-induced hypercalcemia involves the withdrawal of calcium carbonate and calcitriol, and if necessary, fluid therapy,

zoledronic acid, and methylprednisolone, the latter used in specific situations as the first-line therapy for calcitriol-induced hypercalcemia.

Although the pathophysiological mechanism of CAS is not yet clearly elucidated, we can say that the trigger is hypercalcemia due to the imbalance between increased intestinal calcium absorption and the excretion capacity of the kidney (12). This hypercalcemia indirectly increases the reabsorption of bicarbonate in the kidney, causing alkalosis.

CAS is becoming an important public health issue due to the growing number of reported cases. Therefore, clinicians should consider this entity, especially in patients receiving continuous calcium supplements and a vitamin D metabolite, as they are at higher risk. Additionally, it is advisable to maintain close monitoring of renal function and pH in this group to enable early diagnosis and prevent possible complications.

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