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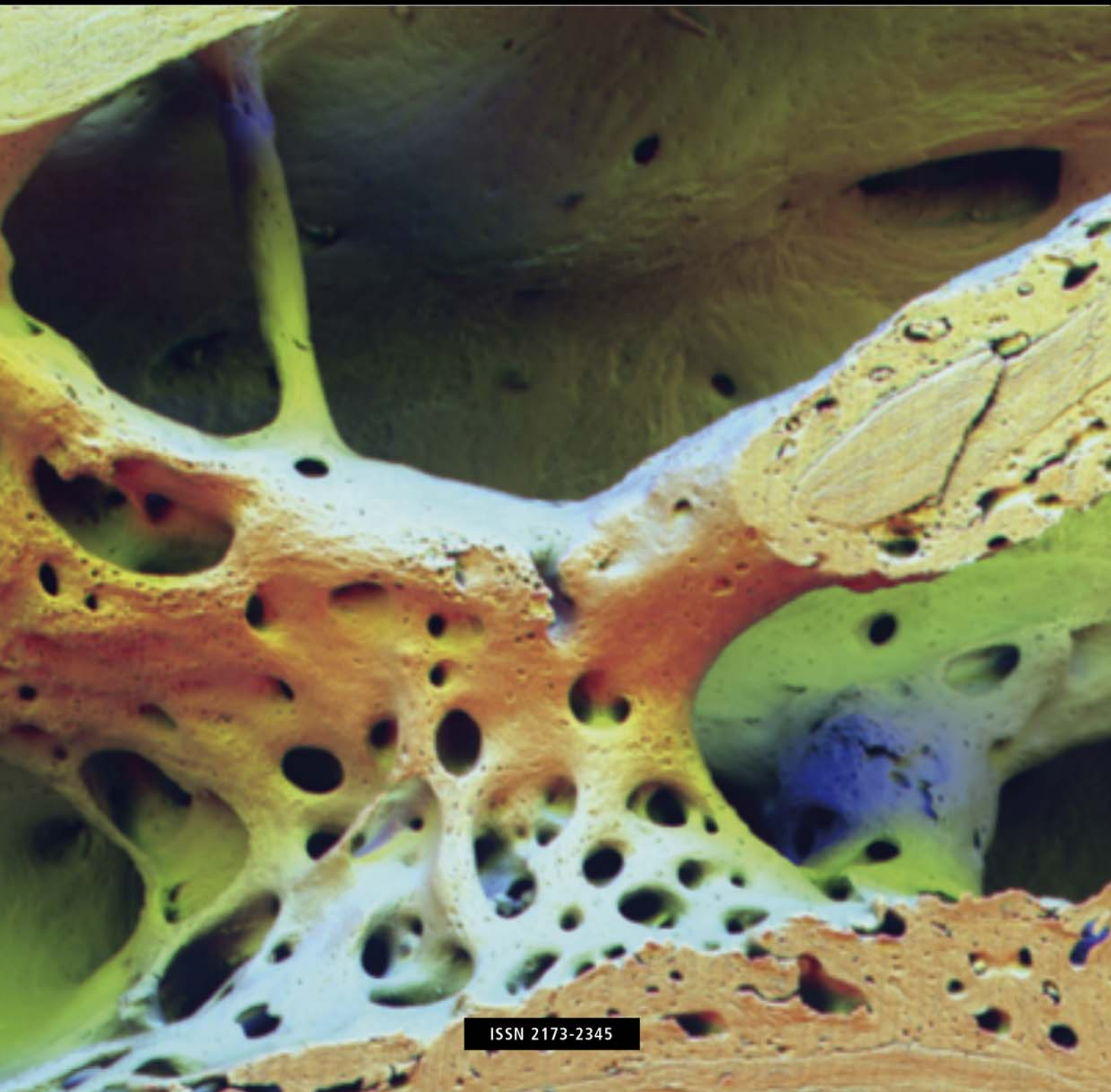

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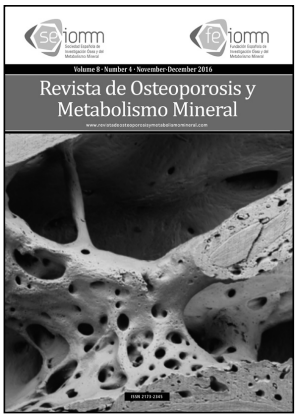

Our cover
Prostate cancer. Metastasis in a vertebral body

Autor:
Courtesy of Professor Alan Boyde. London. United Kingdom

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SUMMARY

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Chronic renal failure, vascular calcification and the RNK/RANKL/OPG system

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Cardiovascular complications are among the most important clinical challenges in patients with chronic kidney failure (CKF). These are frequent processes that present high morbidity and mortality. As an example, around 50% of patients with terminal CRF die from this disease[1].

Renal patients present two types of vascular calcifications: calcification of the tunica media, also called Mönckeberg sclerosis, in which the mineral is deposited within the layer of smooth muscle. The second type is calcification of the intima, in which the calcium deposit occurs after the accumulation of cholesterol under the damaged endothelial monolayer[2]. Calcification of the tunica media, where vascular smooth muscle cells (VSMC) and elastic fibers are found, is not related to cholesterol levels or the existence of atheromatous plaques and causes the hardening and decrease in the arteries' distensibility. Atherosclerotic calcification of the intima may also occur in patients with CRF. In these cases, intimal calcification is associated with the subintimal deposit of lipids and lipoproteins, which may stimulate the development of immune responses, both innate and adaptive, inducing endothelial cells and the VSMC to express inflammatory molecules, which stimulate tumor-infiltrating monocyte/macrophage.

As a result, increased inflammation, oxidized lipids and fibrous matrix secretion in atherosclerotic lesions further accelerate vascular calcification, which eventually leads to atherosclerotic plaque rupture[1-3]. In patients with CKF, both atherosclerotic intimal calcification and tunica media calcification, independent of atherosclerosis, are associated with an increase in cardiovascular mortality compared to patients with CKF who do not present it[4].

Initially this was considered a secondary disorder to the passive deposit of calcium and phosphorus in the vascular wall. However, more recently vascular calcification has been found to be a perfectly regulated process by which VSMC undergo molecular and phenotypic changes. With these alterations they acquire some of the functions that characterize osteo-chondrocitary strain cells[1,2], and lead to the release by vesicular VSMC structures containing hydroxyapatite[5]. In this process of "osteo-chondrocyte transdifferentiation" different factors involved in the differentiation of bone cells, such as Runx2, bone morphogenic proteins (BMPs), RANK/RANKL/OPG system or Wnt pathway would intervene. Furthermore, in patients with CKF and in animal models of this disease, increased vascular calcification is accompanied by a reduction in bone mass, suggesting that the signals involved in bone and vascular wall mineralization may behave differently depending on the tissue microenvironment in which they act[6,7].

CKF is characterized by changes in bone metabolism that, in addition to being detrimental to the skeleton -renal osteodystrophy- favor calcification of soft tissues and vessels. Hypercalcemia and hyperphosphatemia, hyperparathyroidism, increased fibroblast growth factor 23 (FGF23), increased oxidative stress and decreased inhibitors of calcification such as fetuin-A and pyrophosphates could all play a role in the vascular calcification process[1,2,6,7].

Hyperphosphatemia, as well as hypercalcemia, are two of the main factors associated with the development of vascular calcification in CRF[8]. The diet with high phosphorus content increases vascular calcification and reduces bone mass in rats with chronic renal failure. On the other hand, treatments with high calcium and/or phosphate content induce the calcification of VSMC in experimental animals. Although the mechanisms involved in this process have not yet been accurately found, hyperphosphatemia has been shown to induce vascular calcification, favoring osteogenic expression such as Runx2 or BMP2[9,10]. Some authors have shown that, unlike normal vessels, the arteries of CKD patients express Runx2[1,2,9,10]. On the other hand, the uremic serum increases the expression of Runx2 and the calcification of the VSMC. In addition, hyperphosphatemia activates the Wnt pathway, favoring β-catenin translocation in the smooth muscle cell nucleus, thereby stimulating the expression of direct target genes such as cyclin D1, axin 2 and VCAN/versican[10]. Finally, hyperphosphatemia also increases the levels of FGF23, which, together with its co-receptor klotho, may play a pathogenic role in arterial calcification and in the alteration of skeletal mineralization[11].

The role of PTH is also complex. In hemodialysis patients, increased PTH is associated with vascular calcification and, in rats with renal failure, both aortic calcification and loss of bone mass are asso-

ciated with increased phosphorus and PTH[12]. However, in other studies it has been pointed out that PTH is not able to directly induce vascular calcification, but would have a synergistic effect with phosphate, which would be related to increased osteoclastic activity and bone remodeling that this hormone determines. This increase in bone remodeling favors calcium and phosphorus loss from the bone, thus stimulating vascular calcification. It is one of the determinants of the most frequent forms of renal osteodystrophy, osteopathy with high remodeling or secondary hyperparathyroidism. At other times, as with adynamic bone, the low bone remodeling determines an alteration in bone formation and mineralization, with the consequent reduced use of excess calcium and phosphorus, which also favors vascular calcification[13,14].

The increase in oxidative stress observed in patients with CRF would also be closely associated with the development of vascular calcification. As with hyperphosphatemia, this effect would be mediated through the expression of Runx2 in the VSMC[15]. In addition, a recent study carried out in postmenopausal women found that increased oxidative stress was associated with an increased risk of hip fracture, suggesting that there would be an inverse relationship between oxidative stress and mineral metabolism[1,5].

Along with increased levels of calcium and phosphorus, the decrease in some of the inhibitors of calcification, such as fetuin-A and pyrophosphate, which can be observed, can contribute to the increase of vascular calcification in these patients[1,2,6].

In this issue of the Journal of Osteoporosis and Mineral Metabolism, Martínez Arias et al.[16] analyzed the effects of the RANK/RANKL/OPG system on bone demineralization and vascular calcification in CRF. These authors use *in vivo* and *in vitro* models of vascular calcification to verify that rats with chronic renal failure and a diet high in phosphorus present decreased bone mineral density, together with aortic calcifications that are accompanied by an increase in RANKL gene expression and a decrease in OPG. In the tibia of these animals both RANKL and OPG expression increased, although the increase in OPG occurred at earlier stages. In the VCAM, the addition of uremic serum and calcifying medium induced an increase in calcium content and RANKL and OPG expression, while the addition of OPG and the silencing of RANK inhibited this phenomenon. Therefore, these authors' results confirm the RANK/RANKL/OPG axis involvement in the vascular calcification process and probably also in the loss of bone mass that accompanies CRF. This opens the door to new research lines in this area. As the authors[16] comment, there is a great deal of scientific evidence linking the RANK/RANKL/OPG system to vascular calcifications[1,2,7,16-20]. The first derivative of the OPG-null mouse study conducted a few years ago by Bucay et al.[17], who demonstrated that OPG-deficient mice exhibited vascular calcifications, as well as an intense decrease in bone mineral density (BMD) and one High incidence of fractures. It was later found that treatment with recombinant OPG significantly reduced vascular calcification in mice deficient in LDL receptors[18]. On the other hand, the studies carried out in patients with CKD indicate that the levels of RANKL and OPG increase as do those of PTH and phosphate, and it has been pointed out that the increase of Runx2 increases the expression of RANKL in VCAM. In animal models, increased RANKL induces a loss of bone mass and vascular calcification, while the addition of OPG has the opposite effect. The pathway by which RAKL would promote calcification would be through binding to its RANK receptor, with the consequent activation of the NF-kB alternative pathway and the bone morphogenic proteins 2 and 4 (BMP2 and BMP4), favoring the osteogenic transition of the VSMC[1,2,19,20]. On the other hand, RANKL could also act indirectly by stimulating the release of pro-cytokines by macrophages.

Finally, and as might be expected, the Wnt pathway also appears to be involved in this process. We have already commented that hyperphosphatemia would activate this pathway in the VCAM[10]. On the other hand, the expression of sclerostin increases in arteries with vascular calcification. Levels of sclerostin and other Wnt pathway inhibitors, such as Dickkopf-1 (DKK1) or soluble frizzled receptor (SFR), increase as renal function deteriorates and correlate inversely with histological parameters of bone remodeling and with the number and function of osteoblasts[21,22]. It has recently been pointed out that the increase of FGF23, which accompanies renal function deterioration, could also act to inhibit this system[11]. Therefore, sclerostin and other inhibitors of the Wnt system, released into the medium from the vessels, could act to impair the bone structure and retard the mineralization process. These alterations, along with those of the RANK/RANKL/OPG system, hyperphosphatemia and other factors discussed here, could help medical researchers to understand the complex relationship between vascular calcification and bone loss and increased fractures in patients With CRF (Figure 1).

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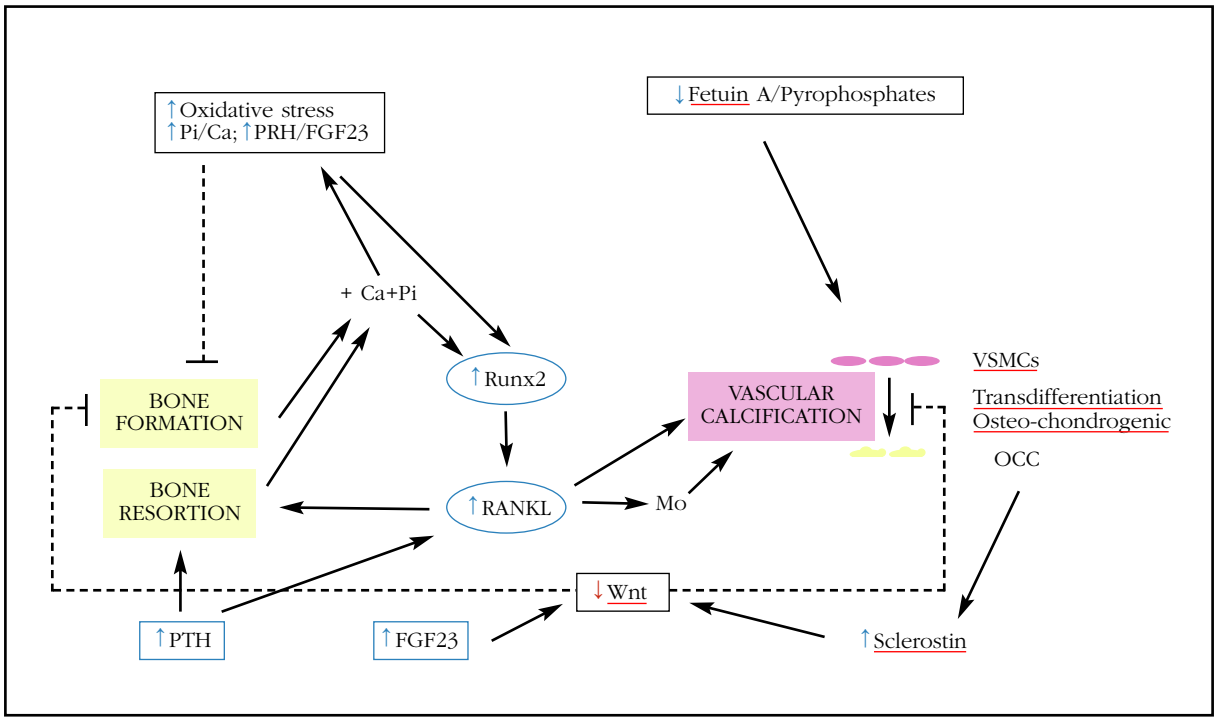
Figure 1. Regulation of vascular calcification in chronic renal disease



Pi: phosphorus; Ca: calcium; Mo: monocyte-macrophage; VSMCs: vascular smooth muscle cells; OCC: osteochondrocyte cells.

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Effect of RANK/RANKL/OPG pathway on bone demineralization and vascular calcification in chronic kidney disease

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Summary

Introduction: In cases of chronic kidney disease (CKD), bone and mineral metabolism changes occur which favor soft tissue calcification. Alterations in the RANK/RANKL/OPG system could also favor vascular calcification, a major cause of morbidity and mortality in CKD.

Objective: In an *in vivo* experimental model of chronic renal failure progression, we assess the effect of CKD on vascular calcification and bone loss correlating these changes in the RANK/RANKL/OPG pathway. An *in vitro* system was used to confirm findings.

Material and Methods: Two models of vascular calcification were used: an *in vivo* rat model with chronic renal failure fed on a diet with different phosphorus content, and an *in vitro* model in vascular smooth muscle cells (VSMC) subjected to different calcifying stimuli.

Results: At 20 weeks, 50% of animals with a diet high in phosphorus presented aortic calcification accompanied by increased aortic expression of RANKL. In contrast, OPG decreased probably as a consequence of an inflammatory component.

At 20 weeks, expression of RANKL and OPG in the tibia increased, while the increase in OPG occurred at earlier stages.

In VSMC, the addition of uremic serum and calcification medium increased calcium content and expression of RANKL and OPG. The addition of OPG and silencing of RANK inhibited this increase.

Conclusions: Our results confirm RANK/RANKL/OPG system involvement in the vascular calcification process.

Key words: *RANK, RANKL, OPG, chronic kidney disease, vascular calcification.*

Introduction

Vascular calcification is a process in which vascular smooth muscle cells (VSMC) and other populations of blood vessel cells undergo a transformation and begin to resemble osteoblasts[1]. This process is regulated in a manner similar to bone mineralization, with several bone proteins being implicated[2-4]. Osteoblasts are cells responsible for the formation of bone that also regulate the activity of osteoclasts and therefore play an important role in the homeostasis of calcium (Ca) and phosphorus (P)[5]. Osteoblasts secrete the NF-κB activator receptor ligand (RANKL) that binds to its receptor (RANK) in osteoclast precursors promoting formation, activation and survival[6-7]. In addition, osteoblasts secrete osteoprotegerin (OPG), which acts as a soluble receptor lure of RANKL and inhibits the binding of this ligand to its transmembrane receptor RANK. There is considerable scientific evidence linking the RANK/RANKL/OPG system to vascular calcifications, which may be an important autocrine/paracrine system involved in the process. The pathway by which RANKL promotes calcification through binding to its RANK receptor with the consequent activation of the NF-κB alternative pathway and bone morphogenetic protein 4 (BMP4)[8] has been implicated in the osteogenic transition of VSMCs[9,10].

Chronic kidney disease (CKD) is characterized by changes in bone and mineral metabolism that favor the calcification of soft tissues and vessels. Alterations in the gene expression of the RANK/RANKL/OPG system could be favoring vascular calcification, one of the main causes of mortality in CKD. It is interesting to investigate the differences in the regulation of the RANK/RANKL/OPG system in bone and vessel in order to design strategies aimed at protecting the bone without having negative effects on vascular calcification.

Therefore, this study aims: a) to evaluate in a rat model the effect of CKD and diets with different P content on vascular calcification quantified by Ca content analysis and bone mineral density (BMD), quantified by bone densitometry; B) to correlate these changes with alterations in the RANK/RANKL/OPG system gene expression in arteries and bones of these animals; And c) to use an *in vitro* system to confirm the findings found *in vivo*.

Materials and methods

In vivo studies:

Vascular calcification model

The protocol was approved by the University of Oviedo's Ethical Committee of Animal Experimentation.

The study was performed with male Wistar rats (n=55) at 4 months of age (350-400 g). Surgical intervention, following inhalation of isoflurane anesthesia, involved inducing chronic renal failure (CRF) (7/8) in a single surgical procedure. Complete nephrectomy of the right kidney and then subtotal nephrectomy of the left kidney were carried out by lateral incision. This procedure preserves approximately one fourth of the renal mass. The rats with CRF were divided into two groups: one, CRF C, fed a standard rodent diet with normal P content (0.6% P, 0.6% Ca, and 20% protein content, Panlab, Barcelona, Spain), and the other, CRF P, fed a diet with high P content (0.9% P, 0.6% Ca, and 20% protein content, Panlab). The study lasted 20 weeks (CRF 20C and CRC 20P), time required to induce vascular calcifications. We also included a Sham group (n=10) that was followed up to week 20. Intermediate evaluations were also performed throughout the study, with sacrifices at 8 and 12 weeks (CRF 8C, CRI 12C, CRI 8P and CRI 12P). Twenty-four hours before slaughter, the rats were housed in metabolic cages and received diet and water *ad libitum*. They were sacrificed using CO_2 anesthesia, and serum samples were taken for analysis. From each rat the abdominal aorta was removed down to the bifurcation of the iliac crests and divided into three portions: the first fragment was used for the extraction of RNA, the second fragment to determine the Ca content, and the third fragment was stored in paraffin for future studies.

At the time of sacrifice the two tibia were removed. The left was preserved in alcohol to measure bone mineral density (BMD). The remaining tibia was frozen at -80°C until processed for the study of gene expression.

Biochemical markers

Serum urea, creatinine, Ca and P were measured using a Hitachi 717 multi-channel automatic analyzer (Boehringer Mannheim, Berlin, Germany). Parathyroid hormone (PTH) was measured by ELISA (Immutopics, San Juan Capristano, USA) following the manufacturer's protocol.

Bone densitometry

BMD was measured in tibia at three levels: proximal octave, seven/eighth distal and total tibia, with a Hologic QDR-1000 dual-energy digital radiological densitometer (Hologic, Bedford, USA) equipped with a specific program for small animals.

Analysis of aortic calcification

Calcification of the rats' abdominal aorta was analyzed by two methods: total Ca content and von Kossa staining.

To determine total Ca content, a fragment of the abdominal aorta (the cm proximal to the iliac bifurcation) was homogenized with an Ultraturrax (OmniHT) in 0.6 N HCl. After shaking at 4°C for 24 hours the samples were centrifuged. The Ca content was determined in the supernatant by the o-cresolphthalein complexone method (Sigma-Aldrich, St. Louis, USA), and the pellet was resuspended in lysis buffer (125 mM Tris and 2% SDS , PH 6.8) for protein extraction and quantification by the method of Lowry (Bio-Rad, Hercules, USA). The Ca content was normalized by expressing as µg Ca per mg protein.

To carry out von Kossa staining, another fragment of the abdominal aorta was included in methyl methacrylate (Sigma-Aldrich). Five 5 mm thick sections were obtained using a Polycut S Microtome (Reicher-Jung, Heildelberg, Germany) and stained following the von Kossa method.

Gene expression study

RNA extraction was carried out by the guanidinium-phenol-chloroform thiocyanate method. DNA copy (cDNA) was synthesized using the high capacity kit (Applied Biosystems, Foster City, USA). The RANK, RANKL and OPG gene expression was analyzed by real-time PCR (qPCR) on Applied Biosystems ABI Prism 7000 equipment. Assay on-demand assays designed by Applied Biosystems employing specific oligos and fluorescent Taqman probes were used for each of the PCRs. GAPDH was used to quantify and normalize the expression of the constitutive gene.

***In vitro* studies:**

Primary culture of vascular smooth muscle cells (VSCM)

VSCM from primary culture of aorta explants from healthy Wistar rats at 2 months of age was used, sacrificing 12 rats and using CO_2 anesthesia. Abdominal aortas were removed and introduced into cold PBS with 100 units/mL penicillin and 100 mg/mL streptomycin (Biochrom AG, Berlin, Germany). After washing abundantly with cold PBS, the aortas were cut longitudinally; The endothelial layer was carefully removed and subsequently cut into fragments (explants) of 2 to 3 mm^2. The explants were plated in six-well culture plates (Sigma-Aldrich) pretreated with fibronectin (10 mg/cm^2; Sigma-Aldrich). Once the explants were placed, 1 mL of DMEM (Dulbecco's Modified Eagle Medium, Biochrom AG) supplemented with 20% fetal bovine serum (FBS) (Biochrom AG) was added. The medium was renewed every 2 days. When the cells reached subconfluency, the tissue fragments were removed and the cells were enzymatically separated (0.25% trypsin and 1 mM EDTA).

Cells were seeded at a density of 10^5 cells per culture dish (Sigma-Aldrich) with DMEM supplemented with FBS (10%). Cells obtained by this method were identified as VSCM by the following criteria: (1) cells grow in the characteristic valley and choline pattern; And (2) immunostaining was positive for alpha-actin (mAb from Sigma-Aldrich).

Cells between passages 2 and 8 were used, using three wells per condition and the experiments were performed in triplicate.

Induction of calcification in VSCM

In order to analyze the uremia-induced calcification and to know the implication of the RANK/RANKL/OPG system, two different conditions were used.

For the first condition, the VSCM cultures were treated with DMEM supplemented with 15% uremic rat serum (a set of 8-week CRF rat sera containing 10.8 mg/dL Ca, 6.7 mg/DL P, and 898 pg/mL PTH). As a control condition DMEM was used with 15% serum from healthy rats (a pool of sera containing 10.4 mg/dL Ca, 3.6 mg/dL P and 25 pg/mL PTH).

In a second condition, to confirm the effect of P, the VSCMs were cultured with calcifying medium: DMEM F12+0.1% bovine serum albumin (BSA) with 2 mM Ca and 3 mM P). DMEM control F12+0.1% BSA was used as condition. In both cases, Ca deposition was determined 4 and 8 days after addition of the stimuli.

The effect of OPG (100 pM), silencing of the RANK receptor (increasing concentrations between 100 pM and 100 nM) was tested in VSCM in which calcification was induced with DMEM F12+0.1% BSA with 2 mM Ca and 3 MM of P.

Gene expression study

We proceeded in the same manner as detailed in the section on *in vivo* studies.

Lentiviral production and infection/RANK silencing by shRNA

The RANK gene was silenced in the VSCM by small forks of RNA (shRNA), which were cloned into a lentivirus-based vector (FSVsi). In it were introduced shRNAs whose target was TTAGCTGAGGATGCTGAGGAT and scramble sequences. All of them were co-transfected with the virion packaging elements (VDV-G) in a 293T cell culture using polyethyleneimine. Infectious particles were produced by culturing the cells 3-4 days in medium for VSCM. The medium was then centrifuged at 1,000 g for 5 min and the supernatant was added to a VSCM culture, being replaced by the conventional medium after overnight incubation. Finally, the VSCMs were collected after 4 days and the silencing of RANK with qPCR and Western Blot was checked.

Western Blot

After transfer, the membranes were incubated for 12 hours with anti-RANK antibodies (1:1,000, Cells Signaling Technology, Danvers, USA), and anti-tubulin (01:10,000, Sigma-Aldrich). Binding of the secondary antibody was detected with the Western Blot detection kit ECL Advance (Amersham Bioscience, Buckinghamshire, UK) and the VersaDoc 4000 (Bio-Rad) imaging system system.

Statistical analysis

For the statistical analysis of the results, the SPSS 17.0 program was used. In the case of variables with normal distribution, the comparison of the treatment groups was performed using ANOVA with the Bonferroni test. In the case of variables with non-normal distribution, the Kruskal-Wallis test was used.

Results

1. Biochemistry

In the groups that received the diet with a high P

content (CRF 8P, CRF 12P, CRF 20P), a slight deterioration of renal function was observed with respect to their controls (CRF 8C, CRF 12C, CRF 20C). Aggravated at 20 weeks (Table 1). In the high P diet group, serum Ca significantly decreased only at week 20 (CRP 20P), while serum P increased in all groups with a high P diet, particularly at 20 weeks of treatment. Parallel to P, PTH increased as treatment time increased, being statistically significant from week 12 and particularly at week 20, where severe secondary hyperparathyroidism was observed (Table 1).

2. Densitometric study

Although there was a slight decrease in the BMD of the groups of animals with high diet in P regarding their controls in all the studied sectors, this was only significant at 20 weeks (Table 2). Losses were predominant at the distal level, where there is a higher content of cortical bone, on losses at the proximal level (Table 2).

3. *In vivo* effect of uremia and P overload on vascular calcification, bone activity and RANK/RANKL-OPG system

Although the Ca content of the aortas of animals fed a normal P-content diet was slightly affected by uremia, administration of a diet with high P content increased Ca significantly in a time-dependent manner with respect to the Sham group. Animals receiving the high P diet increased the aortic content of Ca with respect to their respective controls from week 12, with this effect being magnified at week 20. Despite the generalized increase in aortic Ca content, von Kossa revealed visible calcifications in the aorta in only 50% of the animals with diet with high content in P (Figure 1).

Parallel to the increase in Ca content there was an elevation of RANKL expression in the aorta (Figure 2A). RANK expression did not show any differences along the course of CRI (Figure 2B), whereas OPG decreased in all uremic groups, particularly those receiving a high P diet (Figure 2C).

In the tibia, an increase in RANKL and OPG expression was observed at week 20 of the high P diet group (Figures 3A, 3C). OPG expression also increased in all groups receiving high P diet, noting the increase observed at week 20. In contrast, RANK expression remained similar in all groups.

4. *In vitro* effect of uremia and P overload on vascular calcification and the RANK-RANKL-OPG system

Uremic serum induced a significant increase in Ca content at 4 and 8 days (Figure 4A). There was a significant increase in the expression of RANKL (at 4 and 8 days) and OPG (at 8 days of treatment) (Figures 4B, 4C and 4D).

Calcifying medium-treated VMCV (DMEM F12, 2 mM Ca, 3 mM P) showed a significant increase in time-dependent Ca content (Figure 5A). In parallel increased RANKL and OPG expression (Figures 5B, 5C and 5D).

5. *In vitro* effect of the addition of OPG on calcification induced by uremic serum

To confirm the idea that increased RANKL expression is responsible for the Ca content increase in VSCM treated with uremic serum, 100 pM OPG added to the culture medium, which led to a significant decrease of OPG (Figure 6).

6. *In vitro* effect of RANK silencing on calcification induced by uremic serum

Similarly, silencing of the RANK receptor by the shRNA technique significantly reduced the Ca content of the VSCM treated with uremic serum (Figure 7).

Discussion

CKD, a disease characterized by a progressive loss of renal function, leads to the appearance of mul-

Table 1. General biochemistry in the different treatment groups

	SHAM (n=10)	CRF 8C (n=9)	CRF 8P (n=9)	CRF 12C (n=7)	CRF 12P (n=10)	CRF 20C (n=10)	CRF 20P (n=10)
Urea (mg/dL)	34±9	108±27	137±45	119±29	143±51	100±70	200±70
Creatinine (mg/dL)	0.4±0.1	1.0±0.3	1.4±0,5	1.3±0.4	1.3±0.6	1.5±1.3	2.2±0.8*
Calcium (mg/dL)	11.4±0.6	11.8±0.9	11.4±0.8	12.4±0.6	11.4±2.0	12.5±0.6	11.1±0.4*
Phosphorus (mg/dL)	4.8±1.0	5.7±1.1	10.0±3.7*	6.0±1.9	9.4±3.4*	5.6±0.8	12.1±2.8*
PTH (pg/mL)	22 (4-74)	28 (19-55)	139 (59-933)	62 (27-121)	236 (128-1,113)*	80 (54-115)	1.901 (1,117-2,517)*

CRF 8C, CRF 12C, CRF 20 C: groups of rats fed diets with normal phosphorus content sacrificed at 8, 12 and 20 weeks, respectively; CRF 8P, CRF 12P, CRF 20 P: groups of rats fed a high phosphorus diet sacrificed at 8, 12 and 20 weeks, respectively; *P<0.005 relative to its control group with normal diet in P.

Table 2. BMD values in the tibia in the different treatment groups

	SHAM (n=10)	CRF 8C (n=9)	CRF 8P (n=9)	CRF 12C (n=7)	CRF 12P (n=10)	CRF 20C (n=10)	CRF 20P (n=10)
Proximal T. (mg/cm²)	335±22	307±13	303±24	318±19	302±19	332±18	311±29
Distal T. (mg/cm²)	276±10	243±12	236±15	247±12	239±9	263±13	236±20*
Total T. (mg/cm²)	288±12	256±11	250±14	261±13	252±11	277±12	251±21*

CRF 8C, CRF 12C, CRF 20 C: groups of rats fed diets with normal phosphorus content sacrificed at 8, 12 and 20 weeks, respectively; CRF 8P, CRF 12P, CRF 20 P: groups of rats fed a high phosphorus diet sacrificed at 8, 12 and 20 weeks, respectively; *P<0.001 relative to its control group with normal diet in P.

tiple complications and alterations of the cardiovascular system. In order to simulate CKD, we used the animal model with normal CKD from our laboratory.

According to what was observed in the biochemical markers analyzed, the development of CKD was accompanied by alterations in bone and mineral metabolism which were aggravated by hyper-phosphoremia and the development of secondary hyperparathyroidism. It is well recognized that the latter increases bone turnover, negatively affecting the cortical bone (seven/eighths of the tibia or distal area) more severely than the trabecular bone (octave of the tibia or proximal area), which is corroborated in our animals. Although PTH is able to stimulate the expression of OPG, as demonstrated in the tibia of animals with CRF with severe hyperphosphoremia, this hormone is also capable of inducing the expression of RANKL in osteoblasts[11], and this gene may be responsible for the BMD decrease recorded in the densitometric study.

The RANK/RANKL/OPG system has also been associated with vascular calcification. Initial evidence of its implication in this process derives from the study with null mice for OPG that, in addition to a severe decrease in BMD and a high incidence of fractures, calcifications of the aorta and renal arteries[12].

OPG has the ability to inhibit osteoclastic activity and thus prevent the onset of vascular calcification. In fact, in our *in vitro* model, vascular calcification induced by uremic serum was attenuated by the addition of OPG.

As some authors have described, a direct relationship between calcification and increase of RANK/RANKL/OPG at the bone level has been observed. The decrease of OPG in aortic tissue induced in our animal model by uremia is in line with what has been reported by other authors. While RANKL was clearly detectable in patients with calcified aortic stenosis, OPG levels were not detectable[13]. These reductions of aortic OPG by uremia could be due to the process of inflammation during the calcification that occurs with decreases in OPG[14,15].

Figure 1. A) Ca content in aortas of 7/8 nephrectomy rats fed a diet with normal content in P (0.6%) (gray bars) and high P content (0.9%) (black bars), sacrificed at 8 and 20 weeks. Data represent the mean ± standard deviation. *P<0.05 vs same week with normal P (0.6%). B) von Kossa staining of the aortas in the different treatment groups

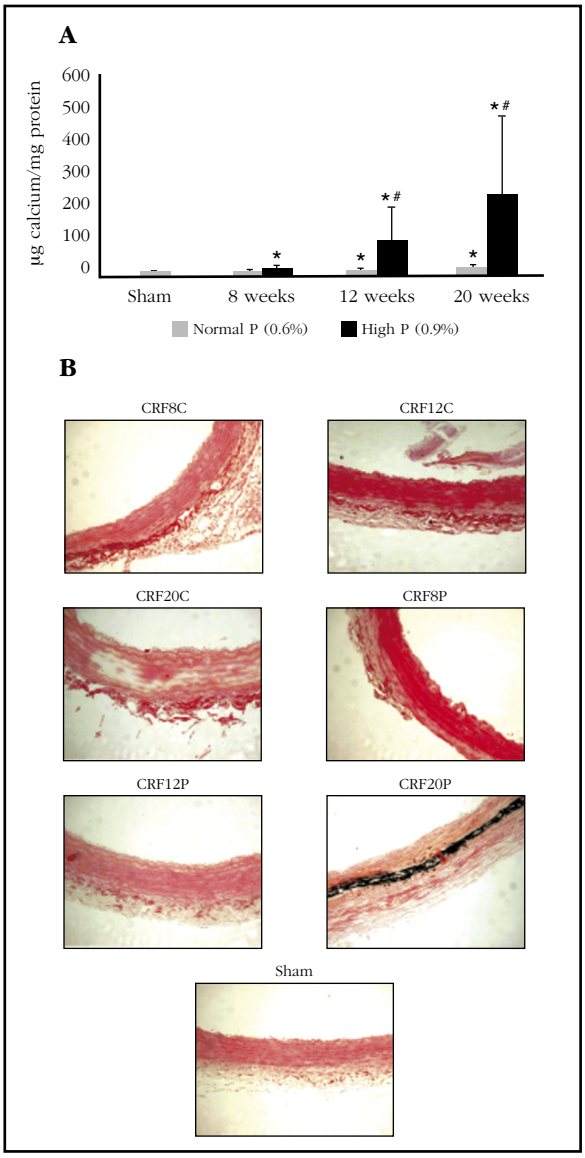


Figure 2. Expression of RANKL (A), RANK (B) and OPG (C) in the aortas of rats with nephrectomy 7/8 fed with diet with normal content in P (0.6%) (gray bars) and high content in P (0.9%) (black bars), sacrificed at 8 and 20 weeks, as determined by q-PCR. Data represent the mean ± standard deviation. *P<0.05 vs Sham group #p<0.05 vs same weeks with normal P (0.6%)

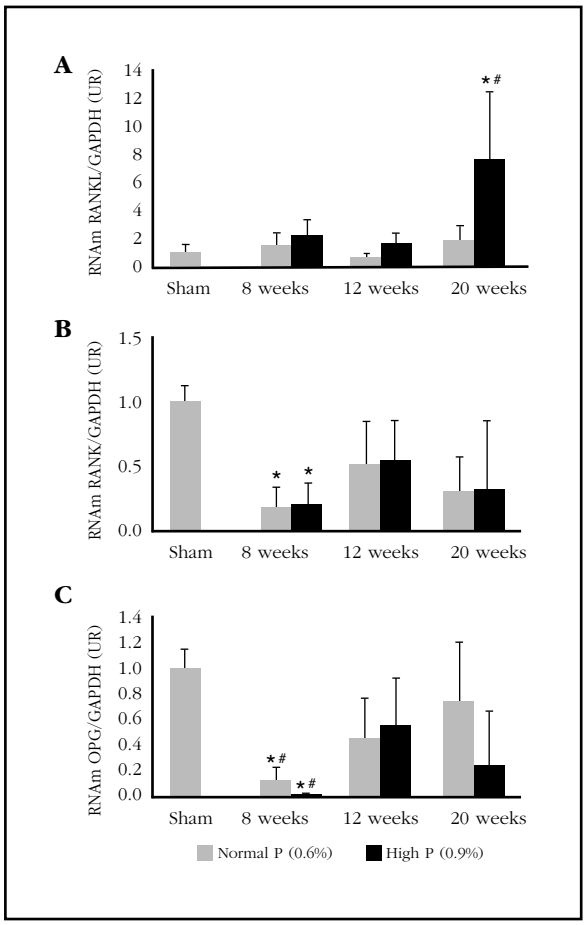
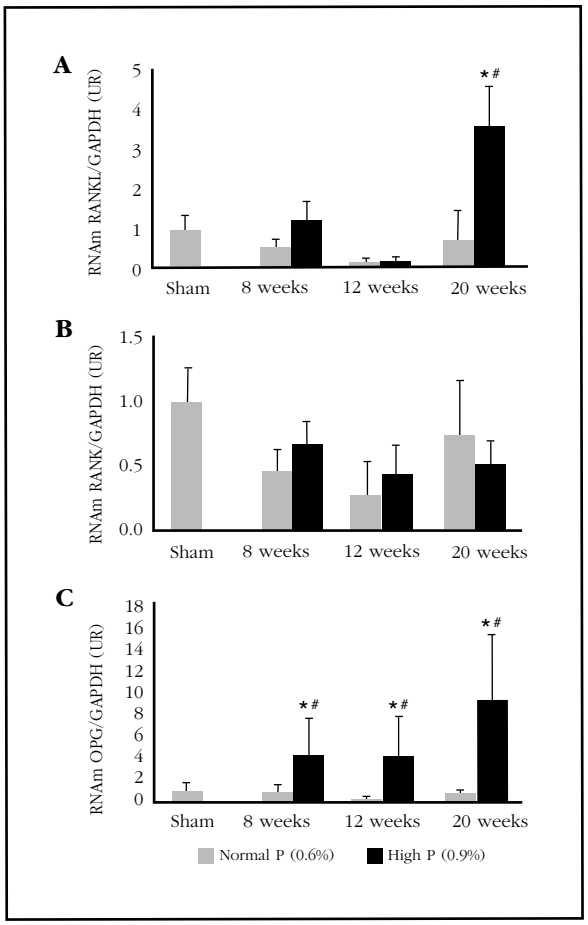


Figure 3. Expression of RANKL (A), RANK (B) and OPG (C) in the tibia of rats with nephrectomy 7/8 fed with diet with normal content in P (0.6%) (gray bars) and high content in P (0.9%) (black bars), sacrificed at 8 and 20 weeks, as determined by q-PCR. Data represent the mean ± standard deviation. *P<0.05 vs Sham group #p<0.05 vs same weeks with normal P (0.6%)



Some authors have described vascular calcification as an active process and regulated by various factors. The VSMC in the early stages of the calcification process undergo a change in its phenotype and begin to express osteogenic markers, which would allow the mineralization of the extracellular matrix. One of these proteins is RANKL, whose expression is abundant in osteoblasts. Both in the aortas and tibias of the rats and in the VSMC there was an increase in the expression of RANKL, aggravated by the increase of P in the diet. In our paper, we show the direct relationship between the increase in calcification and the increase of RANKL. Osteoblasts secrete RANKL, a process that can be reversed by OPG, a protein that sequesters RANKL thus inhibiting the formation of osteoclasts by preventing RANKL from binding to its RANK receptor. The silencing of RANK in our *in vitro* model of calcification with uremic serum inhibited the calcification process by preventing the binding of RANKL to RANK.

Other studies have also shown that RANKL expression increases in calcified areas[16-18], as occurred in the aortas of the animals studied. While in bone an increase of RANKL favors demineralization by an increase in osteoclastic activity, in the vessels it stimulates osteogenesis and, therefore, calcification[19]. In fact, Kindle L et al. suggest that the endothelial cells of the vessel produce a microenvironment favorable to the formation of calcified tissue, stimulating the migration and adhesion of monocytes through the endothelium that can be differentiated into osteoclasts in the presence of RANKL[20]. It has recently been demonstrated that VSCM incubated in a calcifying medium to which RANKL is added increases its Ca content and alkaline phosphatase activity, whereas coincubation with OPG is able to inhibit calcification induced by RANKL[8].

The hypothesis that the RANK/RANKL/OPG system could explain part of the relationship between osteoporosis and vascular calcification is based on multiple epidemiological studies that have revealed the association between bone and vascular metabolism, noting that the decrease in bone mass and Increased fractures were associated with a higher prevalence and progression of vascular calcifications in the general population and in populations at risk[21-26], with the latter being those with CKD.

Figure 4. Ca (A) content and expression of RANKL, RANK and OPG in the VCAM of rats treated with DMEM supplemented with 15% of control serum (gray bars) or uremic (black bars) at baseline (0), 4 and 8 days, as determined by q-PCR. Data represent the mean ± standard deviation. *P<0.05 vs basal; #p<0.05 vs same time with DMEM and serum control

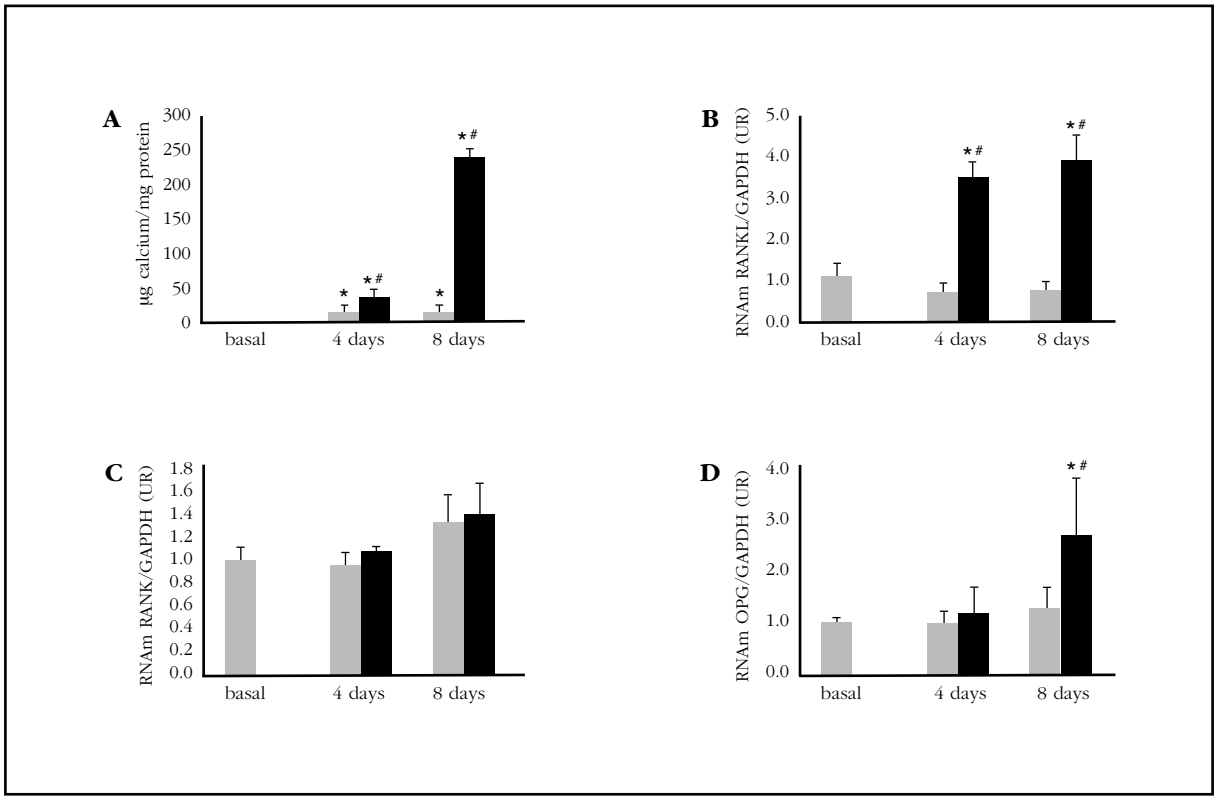


Figure 5. Ca (A) content and expression of RANKL (B), RANK (C) and OPG (D) in the VMCs of rats treated with DMEM conatrol (1mM Ca 1mM P) (gray bars) or calcifying (2mM Ca 3mM P) (black bars). Data represent the mean ± standard deviation. *P<0.05 vs basal; #p<0.05 vs same time with DMEM control

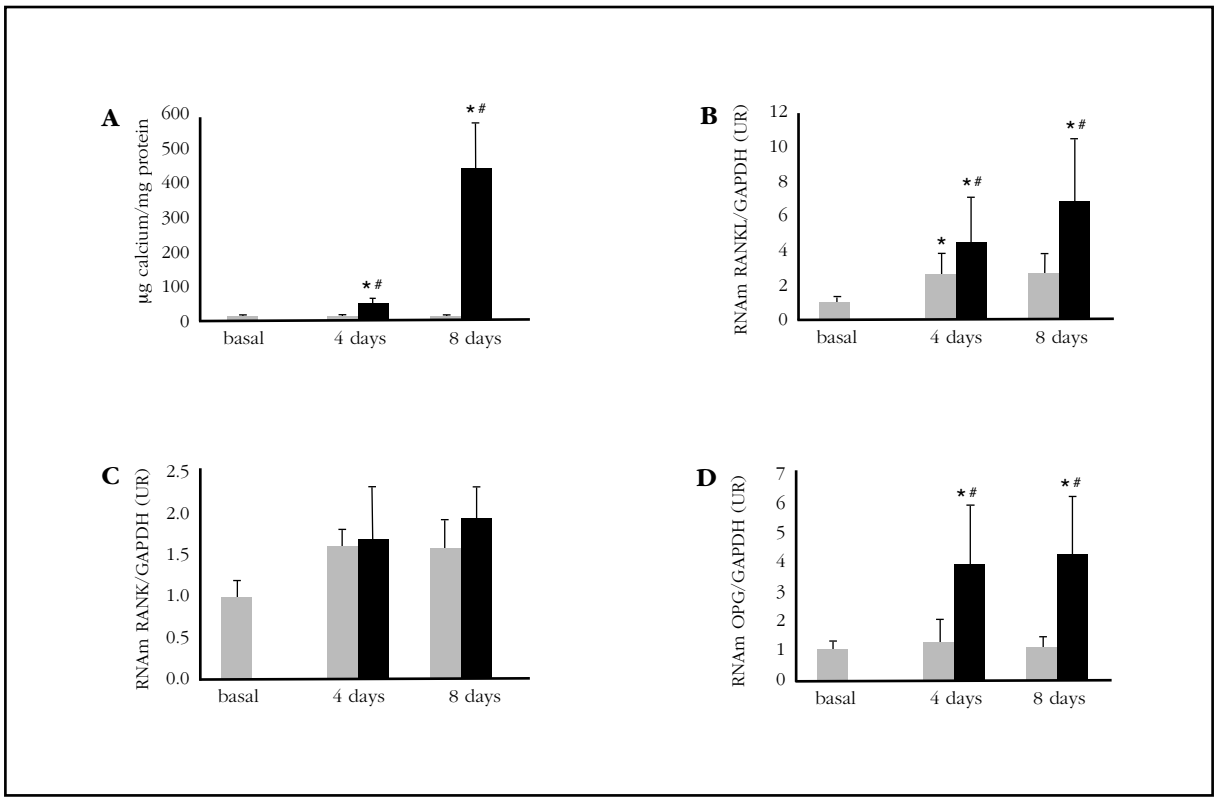


Figure 6. Ca content in VSCM of rats treated with DMEM + 15% control serum (DMEM SC) or DMEM + 15% uremic serum (DMEM SU) with or without 100 pM OPG. Data represent the mean ± standard deviation. *P<0.05 vs DMEM SC; #p<0.05 vs DMEM SU

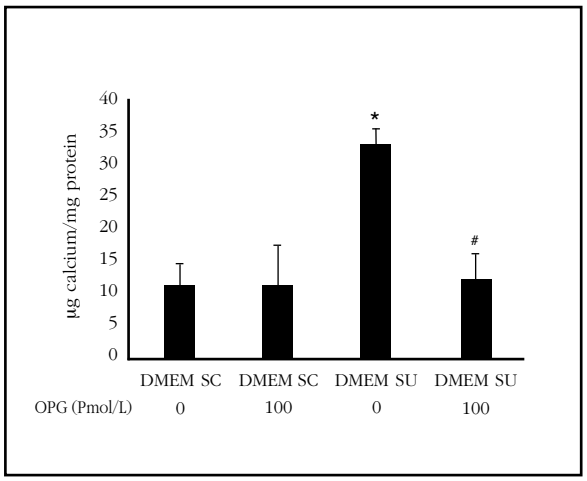
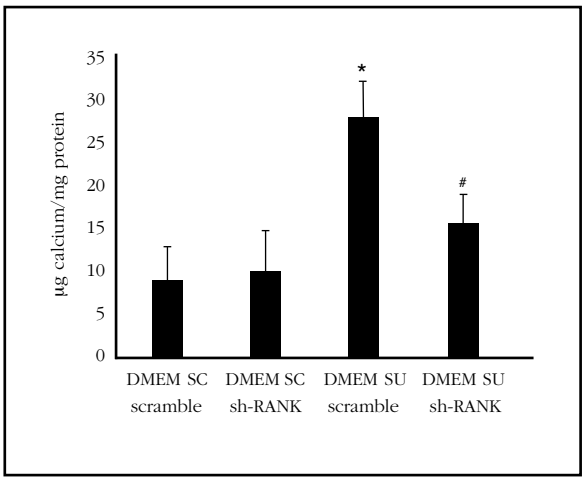


Figure 7. VSCM Ca content of rats transfected with scramble (control) or with sh-RANK with DMEM + 15% serum control (DMEM SC) or DMEM + 15% uremic serum (DMEM SU). Data represent the mean ± standard deviation. *P<0.05 scramble with DMEM SC; #p<0.05 vs scramble with your DMEM SU



The Wnt pathway is an intracellular signaling pathway involved in bone formation. Due to the similarities between bone formation and calcification, it has been suggested that the inactivation of the Wnt pathway could attenuate the calcification process, as has been described by several authors[27-29]. Data from our group, in the same experimental model, have shown an increase in the gene expression of inhibitors of the Wnt pathway in the group of animals with vascular calcification, suggesting a protective mechanism of the progression of calcification[30]. On the other hand, we should not forget that a negative balance of inhibitors of calcification, such as fetuin A, could also favor the calcification process[31,32].

Our *in vivo* results indicate the involvement of the RANK/RANKL/OPG axis in vascular calcification and changes in BMD as a consequence of CKD and of stimuli favoring the former. Moreover, in our *in vitro* model, the addition of OPG as well as the silencing of RANK reduced calcification, indicating that the RANK/RANKL/OPG system acts in this process, opening the doors to new investigations in this area. Because of their importance in the regulation of bone turnover, RANK/RANKL/OPG axis members could be used in the future as useful biomarkers in assessing bone function in patients with CKD.

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

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The association of MMP1 1G>2G polymorphism with aortic valve calcification

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Summary

Introduction: The most common cause of aortic stenosis is active calcium accumulation in the valve cusps, which implies serious clinical consequences. Various extracellular matrix metalloproteases (MMPs) have been implicated in the development of this disease. Therefore, the possible association between a functional MMP1 polymorphism and the amount of calcium deposited on the aortic valve is studied.

Patients and methods: 45 patients undergoing valve replacement were included in the study. The calcium content in valve cusps removed during surgery was determined by computed micro-tomography. DNA was extracted from peripheral blood samples for genotyping the -1607 1G>2G polymorphism of MMP1 by PCR and subsequent digestion.

Results: Significant differences were observed in the calcium content in aortic valves in individuals with different -1607 1G>2G genotypes (p=0.042). Thus, 2G allele carriers (homozygous or heterozygous) present higher calcium levels measured as BMD (p=0.004) as well as BV/TV (p=0.002). The association with BV/TV was independent of sex, age, degree of renal function and anatomy of the valve (p=0.02). BMD tendency (p=0.07) was also observed.

Conclusion: The association between 1G>2G MMP1 polymorphism and calcium content of the aortic valve suggests that the 1G allele would have a protective effect against calcium deposits. These results support the importance of further study to confirm whether this polymorphism could be used as a possible predictor of aortic stenosis development.

Key words: *aortic valve disease, matrix metalloproteinase polymorphisms, microCT, calcium content.*

Introduction

Aortic stenosis degeneration is the most common valve disease in the industrialized countries[1]. Initially considered a passive process, it is now described as an active calcium buildup in the valve cusps, accompanied by changes in the morphology and function of valvular cells, characterized by notable osteoblast differentiation that increases valve stiffness. This leads to a reduction in the orifice opening of the valve and an increase in blood pressure gradient, with serious clinical consequences[2].

There is abundant evidence that implicates extracellular matrix metalloproteinases (MMPs) in this process[3]. MMPs are a large family of zinc-dependent enzymes that exert their function in both pathologic and physiological conditions[4]. Traditionally they have been grouped according to their ability to degrade various components of the extracellular matrix, but also exert functions in other locations. In fact, recently researchers have suggested that they also act on non-matrix proteins and highlight their role in inflammatory processes[5,6]. It has also been found that there is increased expression of MMP-1, -2 and -3 in calcified aortic valves than in normal aortic valves, and the exclusive presence of MMP-9 in diseased valves[7].

The association of MMPs with valve disease has also been studied from the genetic point of view. Thus, the MMP1 1G>2G polymorphism gene at -1607 is reportedly associated with the presence of bicuspid aortic valve anamoly[8]. This same polymorphism has also been associated with levels of bone mineral density in postmenopausal women[9]. Based on the data presented and in order to identify a possible early marker of disease calcific aortic valve, the association of 1G>2G polymorphism with parameters indicative of mineralization in aortic valves from valve replacement was studied.

Patients and methods

Population group

Aortic valves from 45 patients diagnosed with aortic valve disease (stenosis 91%, failure 9%) undergoing aortic valve replacement between April 2012 and May 2014 in the Department of Cardiac Surgery of the Central University Hospital of Asturias were studied. Table 1 shows some features of greater clinical interest are shown, including cardiovascular risk factors.

A current smoker was considered active if that person had smoke during the previous year. An ex-smoker was an individual who gave up smoking more than a year prior and non-smoking to a person who has never taken up the habit. Dyslipidemia was defined according to compliance with any of the following criteria: history of diagnosed hyperlipidemia and/or treated with medication, diet and/or exercise, figures of total cholesterol above 200 mg/dl, LDL cholesterol greater than or equal to 130 mg/ dl, HDL cholesterol less than 40 mg/dl or lipid-lowering therapy. Hypertension was defined as meeting one of the following criteria: history of diagnosed or treated with medication, diet and/or exercise hypertension; systolic blood pressure less than 140 mmHg or diastolic less than 90 mmHg, at least two determinations; or antihypertensive treatment not administered as therapy to anything other than hypertension disorder. The existence of diabetes mellitus was based on the presence of any of the following: accredited medical history of diabetes mellitus, blood glucose greater than or equal to 200 mg/dl fasting in any situation and symptoms of diabetes mellitus, the least two determinations of blood glucose greater than or equal to fasting 126 mg/dl (fasted understood as a period without intake for at least 8 hours) or use of oral hypoglycemic current treatments and/or insulin. The estimation of glomerular filtration was carried out using the MDRD-4 variable equation. The classification of the valve anatomy was made based on intraoperative findings, in addition to the ECG description prior to surgery.

Tissues removed during surgery were treated for 24 h with 4% formaldehyde and after several washings with water, preserved in 70% ethanol at 4° C in the Principality of Asturias Biobank. Peripheral blood sample tube with EDTA, which was processed in the biobank for extraction of genomic DNA was stored at -20° C until use. Patients signed an informed consent form for use of their biological samples and the study was approved by the Ethics Committee of the Principality of Asturias for Clinical Research.

Genotyping

The MMP1 1G>2G polymorphism at position -1607 (rs1799750) was genotyped by polymerase chain reaction and subsequent digestion with restriction enzyme (PCR-RFLP), following a previously reported procedure[10].

Quantification of calcium content in the aortic cusp

Valvular tissue samples preserved in ethanol were analyzed by computerized microtomography (microCT) in a SkyScan 1174 (Bruker, Kontich, Belgium) available at the University of Oviedo Vivarium research center. Images were obtained using 50 kV and 800 µA parameters. 1,300 images of each of the samples with a pitch of 0.3° rotation and an average frame 2 for a 180° scan were obtained. Scanning each lasted 10 to 20 minutes (depending on valve size) using an exposure time of 6,200 ms. flat field correction at the beginning of each scan.

The images obtained were reconstructed with the NRecon (Bruker) software (Figure 1). Correction values of attenuation coefficient, light ray sharpness, smoothness and ring artifacts were the same in all samples. 3D morphometric analysis was carried out using CTAn (Bruker) software. The volume of interest was manually delimited in each of the samples. The threshold used for all of 0.74 to 3.39 was g/cm^3 of bone mineral density (BMD).

BMD parameters and bone volume/total volume (BV/TV) were considered as measures of the amount of calcium deposited.

Statistical analysis

All statistical analyzes were carried out using SPSS version 15.0 software. It was first confirmed that genotype and allele frequencies of polymorphism were in Hardy-Weinberg Equilibrium Model by χ^2 test. ANOVA test was used to compare the mean values of the parameters studied in the different genotypes and then Bonferroni test to discriminate which genotype pairs showed statistical significance. Then, based on these results, the genotypes were grouped into two categories whose average values for BMD and BV/TV were compared by T. Finally test, an adjusted linear regression analysis was performed for variables of sex, age, glomerular filtration rate measured using the MDRD-4 and presence of bicuspid aortic valve anamoly. A p-value <0.05 was considered statistically significant.

Table 1. Clinical and anthropometric characteristics of the study population

Characteristics	Values
Age[a] (years)	69±11
Mens	63%
Smoking	17.4%
Dyslipidemia	43.5%
Hypertension	67.4%
Diabetes	21.7%
MDRD-4[b] (ml/min)	82±28
Bicuspid aortic valve	20%

[a] average ± standard deviation.
[b] glomerular filtration rate in ml/min/1.73 m^2 (mean ± standard deviation).

Results

Individuals are analyzed in Hardy-Weinberg equilibrium for the 1G>2G, polymorphism at position -1607 with a frequency of 0.49 for the minor allele (1G in our population), similar to that of other European populations (dbSNP).

The average values of BMD and BV/TV in population groups defined by different MMP1 polymorphism genotypes at position -1607 and differences found in both variables were calculated, although they were statistically significant only in the case of BV/TV (Table 2).

Post hoc analysis found that homozygous individuals presented significant differences for the 2G allele compared with homozygous for 1G (p = 0.042), with calcium content values similar to those of homozygous for 2G allele heterozygous individuals. Thus, applying a model of recessive for the allele effect 1G, it was found that allele carriers 2G had significantly higher values of calcium content in the aortic valve (3 times in BMD and 2 times the BV/TV) than noncarriers (BMD values of 62.52±10.99 mg/cm^3 in 2G allele carriers ± 8.54 versus 20.08 mg/cm^3 in the 1G allele homozygotes, and values BV/TV 5.44±0.62% in 2G allele carriers versus 2.52±0.59% in homozygotes for the 1G allele) (Figure 2). These significant differences remained for levels of BV/TV after adjustment for sex, age, presence of bicuspid aortic valve and glomerular filtration rate (p=0.021), maintaining the trend, but without being significant, for BMD levels (p=0.073).

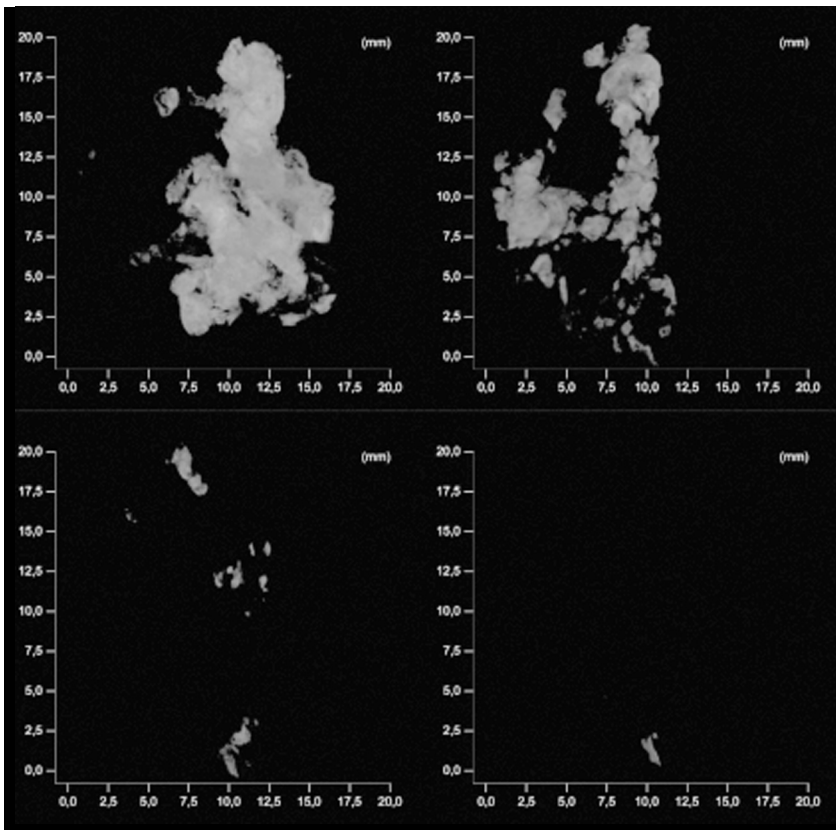
Discussion

This study is the first to describe an association between a polymorphism of MMP1 gene and the amount of calcium in the aortic valves. The literature contains various associations of variants of this gene with other cardiovascular conditions[11-13]. Among these variants, one of the most studied is the 1G>2G polymorphism in the promoter region of the gene, for the insertion allele confers greater transcriptional activity[14] which can have effects on the cell and therefore in the body. Thus, a significantly increased risk of atherosclerosis in the carotid artery in individuals carrying the 2G allele[15] and a greater presence of this allele in patients who had suffered ischemic stroke was observed[16]. However, until now associations of this polymorphism and calcific aortic valve disease had not been described, although the MMPs have been known to play an important role in their physiopathology[17,18].

Two important aspects in the development of disease are calcified aortic valve inflammation and extracellular matrix remodeling[5]. Both are modulated by valve interstitial cells (VIC), which pass from a rest state in which they retain tissue homeostasis, to an activated state which take myofibroblasts[19]. Activated VIC respond to inflammation by secreting, among other factors, MMPs, which will contribute to the accumulation of disorganized fibrous tissue, to keep the valves in a state of chronic inflammation and induce osteoblastic differentiation of VIC. The latter event would promote and accelerate calcium deposition, which would result in reduced function of the valve[20].

The interaction between cells and the extracellular matrix that contains them is essential for the physiology and functionality of the valve tissue and affects the VIC phenotype[3]. The extracellular matrix of the heart valves is made up to 90% collagen and, in fact, excessive deposition of protein, accompa-

Figure 1. Images obtained after analysis by computerized micro-tomography (microCT) of the aortic valve cusps. 4 Examples of valve cusps are shown with varying degrees of calcification



nied by an altered alignment of fibers increases tissue stiffness[21]. Several studies have shown the crucial role of collagen in calcification of aortic valves[22-24]. Specifically, in vitro culture of porcine aortic valve cusps of collagenase treated with increased collagen and simultaneously a decrease in the amount of other components of the extracellular matrix such as hyaluronic acid has been observed[23]. Also, an increase was detected in both proliferation and apoptosis of VIC, which expressed markers associated with a myofibroblast phenotype (alpha-smooth muscle actin) and osteoblast (alkaline phosphatase, osteocalcin and bone sialoprotein) resulting in increased tissue mineralization[23].

MMP-1, also known as fibroblast collagenase, degrades interstitial collagen types I, II and III. Consequently, its increased activity would promote the destruction of collagen, osteoblast differentiation and calcification. The results obtained in this study support this reasoning, associating the highest amounts of calcium in valves with those individuals carrying the allele resulting in increased transcription of the gene and, consequently, a greater amount of MMP-1 protein. Viewed another way, the lack of the protective effect brought about in patients less transcript (provided by the less active allele) facilitating the development of calcification in the aortic valve.

A limitation of our study is the small number of patients included and the fact that it is a cross-sectional study. However, an association with 1G>2G polymorphism has been affirmed, suggesting the protective effect of the 1G allele will necessitate studying larger samples and other population groups in order to ascertain whether this finding could be used in the future as a predictor of calcification and aortic stenosis.

Competing interests: The authors declare that they have no conflict of interest.

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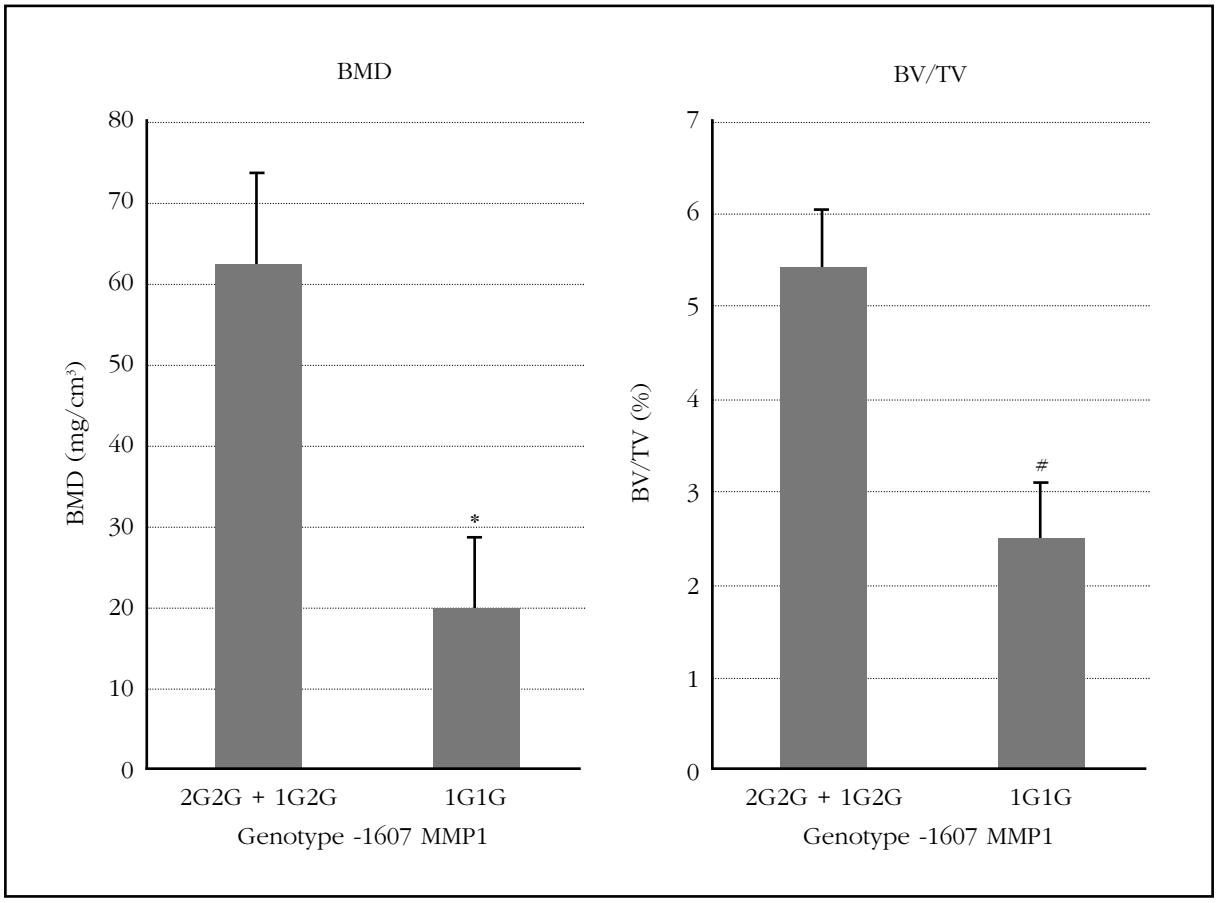
Table 2. Values of BMD and BV/TV for each genotype polymorphism of MMP1 at the -1607 position

Genotype -1607 MMP1	BMD (mg/cm³)	BV/TV (%)
2G2G (N=13)	66.72±65.31	5.87±3.76
1G2G (N=20)	59.79±63.22	5.16±3.51
1G1G (N=12)	20.08±29.59	2.52±2.03
Total (N=45)	51.20±58.96	4.66±3.46
p-valor	0.095	0.033

The data are represented as mean ± standard deviation. P-value obtained by ANOVA.

Figure 2. Values of BMD and BV/TV for MMP1 -1607 genotypes as a model of recessive inheritance for the 1G allele. Average ± standard error is shown. *p=0.004; #p=0.002



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Functional study of promoter gene polymorphisms of sclerostin

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Summary

Sclerostin, encoded by the SOST gene, inhibits the Wnt pathway and, consequently, tends to decrease bone mass. Some polymorphisms of the SOST promoter have been associated with bone mineral density (BMD), but the molecular mechanisms involved are unknown. The aim of this study was to study the functional role of one polymorphism *in vitro*. We cloned the proximal promoter region of SOST gene, containing different alleles at the rs851054 SNP, in luciferase reporter vectors and transfected them into the cell lines HEK-293T, SAOS-2 and HOS-TE85. We did not find significant differences in the transcriptional activity of vectors with either the A or the G allele of the SNP. The co-transfection of vectors expressing RUNX2 and OSX markedly increased the transcriptional activity of the SOST promoter constructs (A allele, 2.5±0.9 fold, $p<0.05$; G allele, 1.9±0.8 fold, $p<0.05$), without significant differences between the rs851054 alleles. Moreover, no allele differences were detected in EMSAs.

In conclusion, the DNA region upstream of the TSS of the SOST gene has a strong promoter activity that is enhanced by RUNX2 and OSX. Frequent allelic variants in this region have been associated with BMD, but the mechanisms involved remain to be elucidated because no functional differences between alleles were detected *in vitro*.

Key words: *sclerostin, gene regulation, polymorphisms, transfection.*

Introduction

Long-term bone mass development is determined by the balance between resorption and bone formation. So, when bone forming activity is insufficient to replace the part destroyed during resorption, it will inevitably lead to decreased bone tissue, characteristic of osteoporosis. Osteoblasts, responsible for bone formation, derived from mesenchymal stem cells, can also lead to other cell types such as chondrocytes or adipocytes. The proliferation and differentiation of osteoblast precursors are controlled by different intracrine, paracrine and endocrine factors. Thus, some transcription factors, such as RUNX2 and Osterix (OSX) are considered essential in the early stages of osteoblast differentiation. The Wnt pathway also plays an important role[1]. Wnt ligands join the cell membrane receptors together and, through various intracellular mediators, modify the expression of target genes that generally promote bone formation and increasing osteoprotegerin (OPG) expression, which secondarily is a decrease in resorption. Wnt receptor ligands are molecular complexes which include at least two proteins: Frizzled and LRP, of which there are several forms[2,3]. As with many other regulatory systems, there are also inhibitors of the Wnt pathway. One of the most widely studied is the sclerostin, encoded by the SOST gene. This gene is expressed preferentially in the osteocytes[4-8]. Being an inhibitor of the Wnt pathway, sclerostin has a negative influence on bone mass. Its role in skeletal biology seems important. Indeed, neutralizing sclerostin antibodies exert a potent anabolic effect on the skeleton, and the SOST gene inactivating mutations cause an exaggerated increase in bone mass[9-11].

In line with the biological role of sclerostin, allelic variants of the SOST gene appear to influence bone mass. Thus, in some studies genome scan (GWAS) and other genetic association studies, we found a link between some common SOST gene polymorphisms and bone mineral density (BMD). Our group also found an association between some polymorphisms located in the promoter region of SOST and BMD in postmenopausal women[12]. The aim of this study was to explore the regulatory capacity of one of these polymorphisms with functional *in vitro* studies. we used vectors in which the promoter of SOST is inserted in front of a reporter gene encoding a protein (luciferase) whose activity can be easily measured. Experiments were carried out to test the binding capacity of nuclear proteins to these regions assessing delay electrophoretic mobility.

Material and methods

Construction of reporter vectors

SOST promoter (1-1440) was cloned from genomic DNA of two individuals with known genotype homozygous for alleles A and G, respectively, of the rs851054 polymorphism (Figure 1). Both fragments were otherwise identical and equal to the reference of the human genome sequences. DNA extraction occurred after informed consent, within a study of genetic factors involved in osteoporosis, authorized by the Ethics Committee on Clinical Research. For amplification of the corresponding genomic fragments, PCR primers designed with the Primer3 program were used which included sequences for the restriction enzymes SmaI and XhoI, to facilitate further cloning. PGL2 vector and amplicon were cut with these enzymes and the fragments were ligated using T4 DNA ligase (NE Biolabs). The vectors were transfected into DH5α competent cells (Invitrogen), which were then grown on agar plates and ampicillin media. Selected colonies were expanded in liquid culture, and then plasmids were extracted and found to contain the correct inserts without mutations, by conventional sequencing. Therefore, in these vectors SOST promoter sequence was found driving transcription of the luciferase-encoding reporter gene. Thus the luciferase activity measurement reflected transcription promoter activity of the SOST allele sequence which was cloned into the vector.

Transfection and analysis of transcriptional activity

HEK-293T cells (a line derived from human kidney) and human osteoblastic lines Saos-2 and HOS-TE85 were transfected with vectors containing the promoter sequences of SOST and a RSV-βGAL control vector, constitutively expressing the LacZ gene, encoding β-galactosidase, in order to normalize the results which depend on transfection efficiency. As a negative control and parallel transfection with empty vector was also performed. For transfection HEK-293T cells 125,000 (50,000 or Saos-2 or HOS-TE85) were seeded into each well of a 24 well plate. After reaching 80% confluency were transfected 500ng vector using Lipofectamine 3000 according to the manufacturer's recommendations (Invitrogen). At 48 hours, the medium was aspirated and the cells with 70 mu.l buffer, after which galactosidase activity (Galacto-Light Plus™ β-Galactosidase Reporter Gene Assay System, Applied Biosystems) was measured and luciferase (Luciferase lysed Assay System, Promega) by luminometry. The co-transfection with expression vectors and OSX[13] RUNX2 was performed following a similar procedure, but ensuring that the total amount of exogenous DNA to be transfected was held constant in all wells trays.

From each transfection, double and triple techniques were performed. Furthermore, each experiment was repeated at least three different times to obtain biological triplicates. The results were expressed as the ratio between luciferase and galactosidase activity in cell lysates.

Analysis of nuclear protein binding

Protein extracts were obtained from 50 x 106 HOS-TE85 cells. To do this, in a lysis buffer was used containing protease inhibitors (50 mM KCl,

0.5% NP-40, 25 mM HEPES, 1.5 pM leupeptin, aprotinin 46.88 µM, 125 µM DTT, 1 mM PMSF). These were centrifuged for 1 minute at 10,000 rpm at 4°C. After washing, the cells were re-suspended in 100 µl of extraction buffer (500 mM KCl, 25 mM HEPES, 10% glycerol, 1.5 pM leupeptin, aprotinin 46.88 µM, 125 µM DTT, 1 mM PMSF) and centrifuged for 5 minutes at 14,000 rpm at 4°C. The supernatant was recovered, the protein concentration was quantified and adjusted to 5 µg/µl.

To test the delay of electrophoretic mobility shift assay (EMSA), oligonucleotide pairs were used, including IR dye labeling 700 in the 5 'end of the "forward" component of each pair (Biolegio). The sequences were as follows:

rs851054 allele A Fwd: AACAGAAACACCTTGGGCCA
rs851054 allele A Rev: TGGCCCAAGGCGTTTCTGTT
rs851054 allele G Fwd: AACAGAAACGCCTTGGGCCA
rs851054 allele G Rev: TGGCCCAAGGCGTTTCTGTT

Prefabricated gels 6% polyacrylamide (Invitrogen) and the Odyssey Infrared EMSA kit were used for retardation. After annealing, the oligonucleotides were incubated with protein extract for 20 minutes, loaded on the gel and were run at 70 V for 60 minutes, after which the images of the gels were captured. In these experiments, if the probes bind nuclear proteins occurs a delay in electrophoretic mobility compared to that experienced by the isolated probe migration.

Results

Constructions SOST promoter sequence showed high transcription activating capacity by increasing levels of luciferase expression up to 1,000 times compared with empty vectors. The activity was apparently higher in HEK-293T cells than in the other lines (Figure 2).

However, no significant differences between the two alleles SOST promoter in any of the cell lines tested. In fact, the ratio between the transcriptional activity of alleles A and G (Ratios A/G) in Saos-2 and HOS TE85-293T cells were 1.3±0.7, 1.0±0.4 and 0.8±0.7, respectively. None of the three was significantly different from the unit (Figure 2).

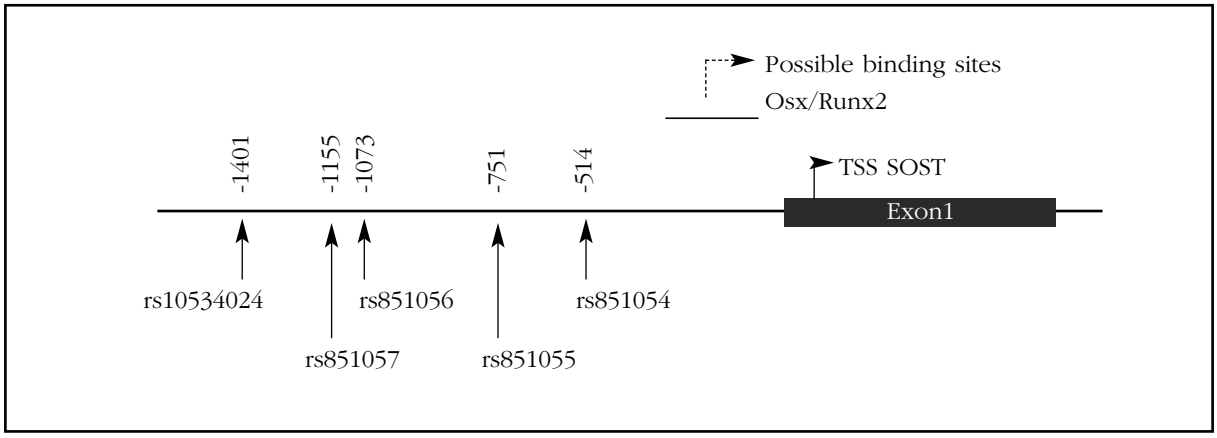
The co-transfection of vectors expressing constitutively RUNX2 and OSX increased transcriptional activity of the SOST promoter (on average 2.5±0.9 times above baseline for allele A and 1.9±0.8 times to the G allele; both $p<0.05$). This increase was also be independent of which allele was present in the SOST promoter (Figure 3).

Furthermore, in experiments delay electrophoretic mobility see that the region where the polymorphism lies was able to bind nuclear proteins, presumably with regulatory function without us to observe differences between alleles (Figure 4). Therefore, no further studies were performed to identify the nature of these proteins.

Discussion

The role of sclerostin in bone biology is undeniable, as revealed by the sharp BMD increase observed after administration of romosozumab, a monoclonal antibody that blocks sclerostin action, and excessive bone mass experienced by patients with sclerosteosis or Van Buchem syndrome or carriers of rare mutations that induce a rare disease such as loss of SOST gene function[14]. Although the evidence is limited, several studies suggest that frequent allelic variants of the gene can also influence, to a lesser extent, BMD and osteoporotic fracture risk. In fact, in a study of genomic sweep with a large sample size of rs4792909 polymorphism association was found with BMD[15]. This polymorphism is located in an intergenic region, being the closest SOST gene, located at about 40 kb. On the other hand, several groups, including ours, have analyzed the relationship of some common polymorphisms in the promoter region of SOST with BMD. So, we found a significant association of rs851054 and rs851056 polymorphisms, only separated by 560 bp and members of the same haplotype block with BMD of the spine in women12. Some authors have confirmed the association of the polymorphism in the SOST promoter with BMD in other population groups[16-20]. On the other hand, in a recent study involving a small group of patients, an association of rs851054 alleles with the expression of SOST in bone tissue was found[21].

Figure 1. Schematic of the promoter region of SOST gene and location of some common polymorphisms (minor allele frequency greater than 5%)



The main objective of this study was to explore the potential functional impact of one of these polymorphisms and to analyze their influence on the transcriptional activity of the SOST gene promoter. Transfection experiments with reporter vectors and delay in electrophoretic mobility did not reveal differences between the allelic variants studied. There are several limitations of the models used and numerous reasons that could explain these negative results. First, it could be argued that the *in vitro* model does not adequately reflect the *in vivo* situation. For example, it cannot be excluded that the regulatory activity requires genomic regions with activity *enhancer*, promoter and far not included therefore in the region cloned into the vectors. On the other hand, at least theoretically it might be thought that the difference in allele activity is only expressed in response to regulatory factors in certain cell types such as osteocytes, but are absent in the cell types used in our experiments. Second, it appears that the association with BMD might rely on other common polymorphisms in linkage disequilibrium with those studied here. If so, they should be in distant, remote regions of the promoter, since in this region there is a strong linkage. In this regard, it should be noted that there is a regulatory region of SOST located at about 50 kb, containing some polymorphisms linked inconsistently with BMD[20,22]. This region is capable of fixing MEF2C transcription factor and plays an important role in regulating SOST because their lack causes the Van Buchem disease phenotype in humans and increased bone mass in murine models[23,24]. A third possibility is that the association with BMD depends on some low frequency polymorphisms located in the promoter region. Finally, polymorphisms might act through epigenetic mediators that cannot be adequately recapitulated in *in vitro* models. Further studies are thus required including the systematic analysis of the variants of the 5 ' and 3' of the gene and others which are associated with considering the tridimensional structure of chromatin and mutagenesis studies *in vivo* to clarify the mechanisms by which these allelic variants may modulate the activity of sclerostin and associate with differences in bone mass[25].

Our study confirmed previous results indicating that RUNX2 and OSX increase the expression of SOST in humans[13]. This indicates that these transcription factors have a complex role in the osteoblastic line. On the one hand, they have a well-established role as determinants of the early stages of differentiation of mesenchymal cells into osteoblasts and enable an adequate number of bone-forming osteoblasts. They also promote SOST promoter activity. Therefore, in cells capable of expressing this gene, such as osteocytes, the secretion of sclerostin could promote and thus help prevent exaggerated bone formation. In any case, the SOST promoter variants do not appear to influence the response to these factors.

In conclusion, in this study we have confirmed that the region located before the start of translation of the gene SOST has potent promoter activity, which also induced transcription factors RUNX2 and OSX. Frequent variants of this region have been associated with bone mass, but the mechanisms involved are still unknown, since the alleles show no differences in *in vitro* transcriptional activity.

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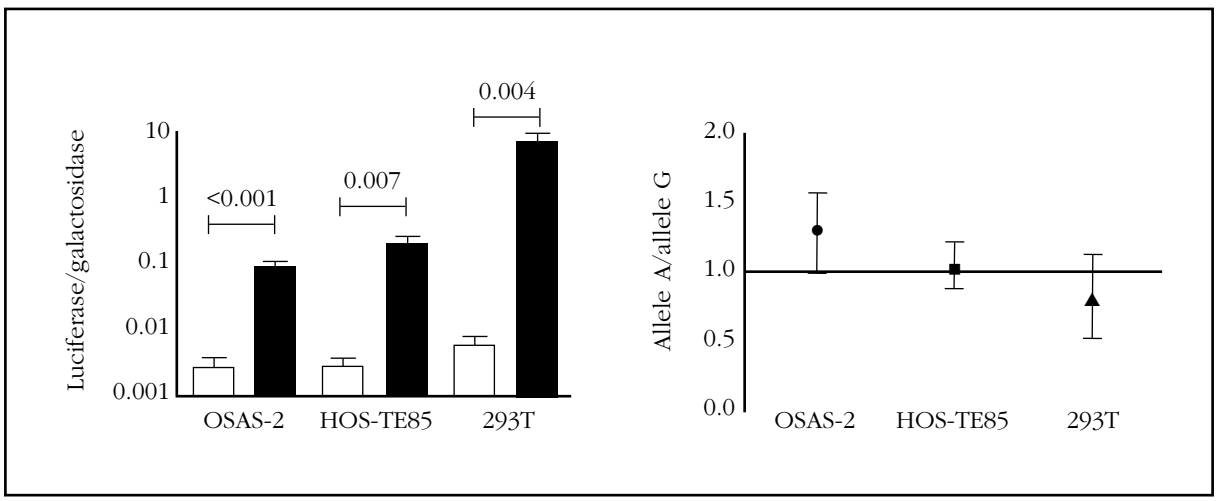
The authors would also like to express their grateful to Dr. Svante Paabo for generously supplying Runx2 expression plasmid.

Figure 2. Transcriptional activity of the promoter sequence of SOST after transfection into osteoblast-like cells (HOS-TE85 OSAS) and HEK-293T line. In the left slide all the experiments (black bars, vectors with promoter sequences SOST white bars, empty vector) are represented; in the right, the ratio of the activity of the constructs with the A and G alleles in each of the experiments. the average values of 5-6 independent experiments performed in duplicate or triplicate are shown. The vertical lines indicate the standard error



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

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Figure 3. Comparison of the transcriptional activity of the vectors A and G alleles in HEK-293T cells in basal conditions and after co-transfection of expression vectors Runx2 and OSX

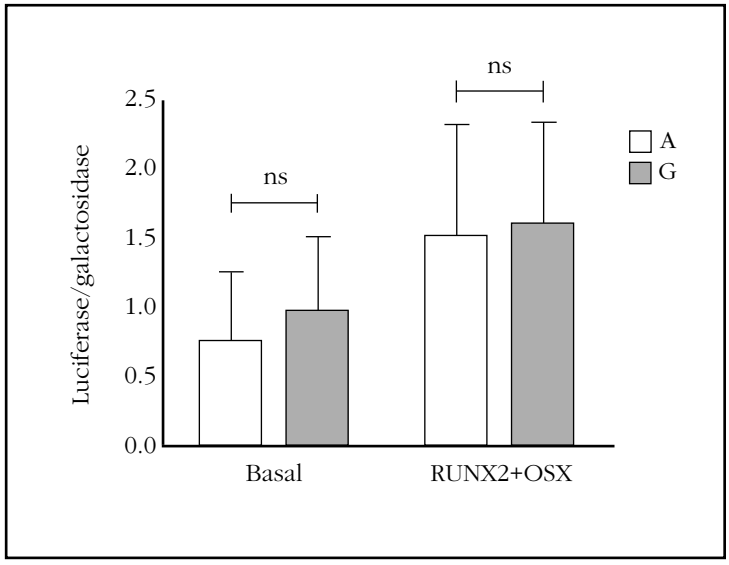
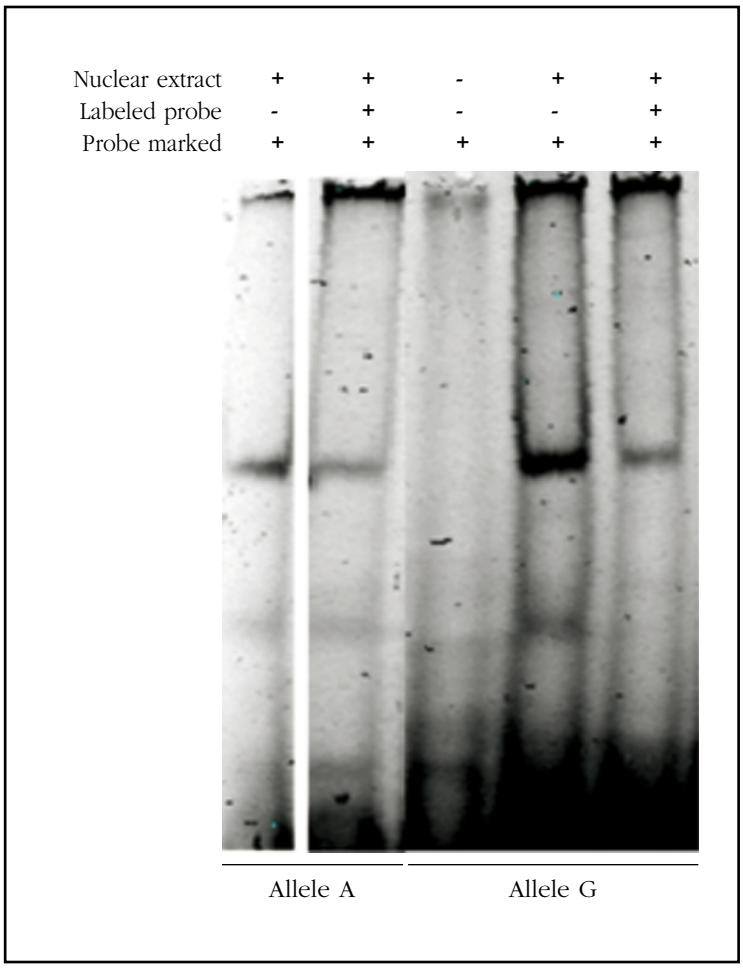


Figure 4. Test of delay in electrophoretic mobility. The onset of delay band is observed when the nuclear protein extract, whose intensity decreases by adding unlabeled probe is added. There is some difference in band intensity between alleles A and G, parallel to the signal strength of the undelayed probe. However, in several experiments no consistent difference between the allele probe and the probe was observed with the G allele



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Prevalence of low levels of vitamin D in patients with breast cancer who live in Northern latitudes 21-22º

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Summary

Objective: Vitamin D has been involved in various diseases, including cancer. Several studies have linked vitamin D levels with breast cancer. The aim of our study was to establish the importance of adequate vitamin D concentrations to prevent breast cancer.

Materials and methods: The study included 76 patients. Dietary habits, sun exposure, body mass index (BMI), and skin type were evaluated. Vitamin D determination in serum was measured by liquid chromatography. Vitamin D receptor pleomorphism was analyzed by immunohistochemistry.

Results: Vitamin D ingestion was deficient in 18 patients and 22 controls; and sufficient in 6 patients and 30 controls, odds ratio of 4.09, confidence interval 95% 1.04-11.0, (p=0.016). Sun exposure was present in 9 patients and 15 controls; 15 patients and 37 controls had less sun exposure or used protection. Two patients and 13 controls had normal levels of vitamin D (30-60), two patients and 26 controls had low levels (20-30), and 18 patients and 12 controls had very low levels (<20). Odds ratio for patients with vitamin D serum levels of 20 ng/mL or less, or higher was 9, CI 95% 2.95-27.5, (p<0.001). These levels were independent from BMI.

Conclusion: Low concentrations of vitamin D are strongly related to breast cancer in a region with high solar exposure. More studies are needed to confirm this relationship.

Key words: *breast cancer, vitamin D, risk factor, sun exposure, diet, skin type.*

Introduction

Vitamin D's importance in maintaining bone mineralization has been recognized for decades. Currently, 30 to 60 ng/mL plasma concentrations of vitamin D have been associated with improvement in other conditions such as hypertension, cardiovascular disease, diabetes, autoimmune diseases and cancer[1].

Breast cancer is the leading cause of cancer death in women in the world[2]. In 2012 an estimated 1.67 million new cases were diagnosed worldwide[3]. This is a significant increase if compared with the 12,433 new breast cancer cases in women aged 40 to 59 years, according to Mexico's National Register of Malign Tumors in 2003. The rate of the disease in Mexico is 18.7 per 100,000 women aged 25, an increase of 49.5% over the past 20 years[4].

Due to the global impact of breast cancer, numerous efforts have been made to identify risk factors and thus develop preventive measures.

A recent study of 44,778 pairs of twins with cancer identified a contribution of approximately 5% of inheritable genetic factors in cancer development. This study suggested that between one and two thirds of cancer cases could be prevented with dietary factors[5]. So efforts have been made to link low vitamin D levels with low sun exposure and particularly breast cancer in women living in Northern latitudes[6-8]. Other epidemiological studies have even shown that high levels of metabolites of vitamin D are consistently associated with decreased risk of breast cancer[9], while low concentrations were significantly associated with negative characteristics of breast tumors, such as tumor size or higher grade[1]. Besides epidemiological findings have demonstrated that breast tissue contains receptors for vitamin D, and several polymorphisms of the gene encoding this receptor have been associated with worse prognosis[10,11].

For most people, between 80 and 90% of circulating vitamin D reserves are derived from accidental exposure to radiation solar[12], which is limited in areas where such radiation is low. That inadequate sun exposure is also due to cultural habits, such as clothing and public health recommendations which are aimed at preventing neoplastic growths such as skin cancer, for example[13]. It is also worth considering that, despite the existence of vitamin D enriched milk, fatty fish and cod liver oil, food sources containing this vitamin are limited, and in many countries, dairy products are not fortified with vitamin D[13].

To confirm the link between low levels of vitamin D and breast cancer, we conducted a case-control study in women living in a region that receives high doses of UVB radiation throughout the year. We tried to eliminate confounding variables by evaluating vitamin D intake, body mass index, family history, hormonal history and the circulating level of vitamin D receptor. Our hypothesis was that vitamin D concentrations would be significantly lower in women diagnosed with breast cancer than in those of the control group.

Material and methods

Study population

We designed a case-control study with information on the population of Aguascalientes, a state in Mexico, located between 21 and 22° north latitude with an average temperature of 19.2°C and receiving a daily dose of between 6 to 7 kWh/m^2 of solar energy[14]. In 2010, the population of Aguascalientes was 1,184,996 inhabitants (National Institute of Statistics, Geography and History of Mexico, INEGI), and in 2012, 150 new cases were diagnosed and treated for breast cancer[15].

The protocol was submitted to the Institutional Ethics Committee on 22 December 2011, adopted on 26 January 2012, and registered with the Federal Commission for the Protection against Sanitary Risk of the Mexican Health Ministry (COFEPRIS) on 19 December 2012 (CAS/OR/01/CAS123300410D0034-3789/2012). All the study procedures were carried out in accordance with the Declaration of Helsinki. All the study participants signed informed consent forms.

Patients in the case group were included if they had breast cancer confirmed by histopathological diagnosis prior to systemic therapy between March 2012 and March 2013. We included two controls for each woman, those who had negative mammogram results (BIRADS 0-2 confirmed by a central radiologist) or benign biopsy, they were matched by age and place of residence in the previous three years. Patients and women in the control group were referred by Oncology Services Advanced Medical Hospital with Central Medical Surgical Area General Hospital Number 1 of the Aguascalientes Delegation of the Mexican Social Security Institute; General Hospital Institute of Health and Social Security Workers in Aguascalientes State; and the Women's Hospital. All patients participating in the study were evaluated between 7 and 15 days after cancer diagnosis through a complete medical history, including eating habits skin type and sun exposure. For all participants, records of vitamin D (25-OH) in serum were taken. Two patients declined to participate in the study. None of the controls refused to participate in the study.

Exclusion criteria for both groups were: previous diagnosis of hyperparathyroidism, intake of vitamin D supplements prescribed or over the counter, existence of previous burn injury have needed a skin graft, intestinal malabsorption, hormone replacement therapy thyroid, neoplasia other and patients with altered levels of serum calcium, phosphorus and magnesium.

All cases and controls were given two separate interviews, one to evaluate the risk factors of breast cancer and sun exposure habits, and the second for a nutritional intake survey of vitamin D. Risk factors for cancer breast are included in the breast Cancer Risk Assessment Tool[16], but in our study we excluded people of color because this model is designed only for white women.

Habits solar radiation were evaluated by a questionnaire on the use of sunscreen, clothing

with long sleeves, radiation exposure by occupation or recreation (or both), and the time and duration of exposure to solar radiation.

In our study we determined the serum levels of calcium, phosphorus and magnesium, and vitamin D (25-hydroxy vitamin D_3) and vitamin D receptor (VDR). The role of vitamin D in calcium homeostasis was assessed using biomarkers as levels of parathyroid hormone, calcium absorption and bone mineral density[17].

As it has shown that adequate intake of calcium and vitamin D reduces significantly bone loss[18], a DXA bone density scan of the lumbar spine and proximal femur was carried out in all patients to determine chronicity in vitamin D deficiency.

Tumor tissue samples were taken from cancer patients to be examined by one pathologist (ARP).

All participants (cases and controls) were interviewed by one of the researchers (RGF/SPJ) who notified the women of the test results. Those who had abnormal levels of vitamin D or bone density diagnosis of osteopenia or osteoporosis were referred to their primary care physician to prescribe treatment.

Determination of vitamin D and its receptor

Measurement of 25 (OH) vitamin D was carried out using liquid chromatography. Pleomorphism receptor Vitamin D was analyzed by immunohistochemistry. The VDR gene polymorphisms (Bsm-1, Fork-1) was formed by cells taken from peripheral venous blood genomic DNA (5 ml blood stored in tubes containing ethylenediaminetetraacetic acid).

The DNA was extracted by Lahiri and Numberger method[19]. Genomic DNA (100 ng) was amplified by PCR technique under standard conditions: 1.5 mM MgCl2, 20 µM dNTPs, Taq DNA polymerase 1 IU, final volume of 50 µl.

Statistic analysis

The odds ratio was used to estimate the association between low levels of vitamin D and breast cancer as study participants were selected based on the presence or absence of breast cancer, and not for their vitamin D levels. To quantify the accuracy of the association, a confidence interval of 95% was calculated, and to assess the risk weighted in the presence of confounding variables we used the Cochran-Mantel-Haenzel test.

Results

Between March 2012 and March 2013, a total of 76 women were included in the study. All of them met the inclusion criteria and none were excluded because of serum electrolyte abnormalities.

Of these, 24 had confirmed breast cancer aged 22-79 years (median 50.5 and 53.6 average), and were included in the case group diagnosis. The remaining 52 women, aged between 24 and 67 years (median 51 and average of 51.09), were assigned to the control group.

The risk of breast cancer was analyzed by modifying the Breast Cancer Risk Assessment Tool (with ethnicity factor removed); the 5-year risk was similar in both groups.

Sun exposure, at least 30 minutes a day, was present in 9 patients and 15 controls; while 15 patients and 37 controls had less sun exposure or used adequate protection (odds ratio=1.48; confidence interval (CI) 95%: 0.53 to 4.11; p=0.625) (Table 1).

Vitamin D intake was evaluated and classified as deficient in 18 patients and 22 controls, and sufficient in 6 patients and 30 controls (odds ratio=4.09; 95% CI 1.04 to 11.0; p=0.016) (Table 1).

Six patients and 20 controls had normal BMI (18.5 to 24.9), 6 patients and 21 controls were overweight (BMI 25 to 9.9); 12 patients and 10 controls were classified as obese (BMI of 30 or more) (odds ratio=4.42; 95% CI 1.51 to 13.1; p=0.01) (Table 1).

Two patients and 13 controls had normal levels of vitamin D (30-60 ng/mL); 2 patients and 26 controls had low levels (20-30 ng/mL); and 18 patients and 12 controls had very low levels (<20 ng/mL) (Figure 1). Looking at the distribution, we calculated the odds ratio for patients with serum levels of vitamin D of ≤20 ng/mL compared to those with levels above 20 ng/mL, with 9 (95% CI: 2.95 to 27 5; p<0.001) (Table 1).

Bone densitometry was normal in 40.4% of women in the control group, while they were only 20.8% of patients with breast cancer; 16.7% of cancer patients and only 9.6% of the control group were classified as osteoporotic. These findings suggest that there were low levels of vitamin D for a long period of time; however, bone mineral density was not related to current vitamin D levels.

The BSM-1 receptor polymorphism of circulating vitamin D in blood was positive in 4 cases and 5 controls, but was not associated with circulating levels of vitamin D; while Fork-1 polymorphism was positive in 3 control and in one case, and it was not related to circulating levels of vitamin D.

In assessing the risk factors analyzed and breast cancer in our patients we found a positive association. In other words, the risk of breast cancer is 1.48 times higher in women with less than 30 minutes of daily sun exposure, compared with women with sun exposure of 30 minutes or more. Similarly, the risk of breast cancer is 4.09 times higher in women with deficient intake of vitamin D, compared to those in whom it was determined that the intake of vitamin D was sufficient. The risk was 4.42 times higher in women with obesity or overweight and 9 times higher in patients with serum vitamin D levels less than or equal to 20 ng/mL. All these associations, except less sun exposure 30 minutes a day, were statistically significant according to the Chi-square test.

To avoid confusion, we performed a multivariate analysis using the Cochran-Mantel-Haenzel test. The Chi-square with one degree of freedom was significant with a value of 18,863 (p=0.000014), indicating that the association of breast cancer with low levels of vitamin D was independent of the other variables analyzed.

Discussion

Evidence of the relationship between vitamin D and breast cancer has significantly increased in recent years. Garland and Garland suggested the importance of exposure to solar radiation to explain the geographical variation in the frequency of breast cancer[20]. Although several recent studies identified a low or no relationship between sun exposure and breast cancer in women living in regions with low sun exposure, they also found that lower risk is greater in regions with high solar and intermediate irradiation areas with intermediate sun exposure[8]. Similarly, Ingraham emphasizes that in Norway a positive effect on survival in women with breast cancer diagnosed in summer or autumn has been found, when vitamin D levels are higher[21].

These findings concur with those which have demonstrated decreased synthesis of vitamin D in the winter in regions with low sun exposure in the United States[8]. Therefore, it is noteworthy that in our population we found that very low vitamin D levels (<20 ng/mL) are closely related to breast cancer in a region with considerable sun exposure throughout the year, which contrasts sharply with the control group. In our population, this association was independent of BMI, skin type, exposure habits/sunscreen or intake of vitamin D.

This finding confirms the hypothesis that low levels of vitamin D are associated with the development of breast cancer, but does not explain the cause of these serum levels[22]. Another study in Mexican women, which found a 47% reduction in the risk of developing breast cancer in those with serum vitamin D above 30 mg/mL, attributed low levels of vitamin D to limited sun exposure due to the indoor work activity, to lack of outdoor recreational activity, hyperpigmentation of the skin and because they actively avoided sun exposure[23].

One significant aspect for confirming vitamin D's potential role in the development of breast cancer is that aging and estrogen deficiency are also associated with low vitamin D levels. The former reduces the production of skin cholecalciferol, while the latter decreases the metabolic activation of vitamin D. Therefore, postmenopausal women, predominantly white, with breast cancer are at higher risk of vitamin D deficiency, compared with younger women[24], but equally in this study we found no significant difference related to menopausal status.

Two important findings are expressed in VDR breast tissue and there is a greater disease-free period in women with breast tumors with positive VDR, compared to those with tumors negative VDR[25]. The ligand VDR is the metabolite 1,25-hydroxyvitamin D3 (1,25 $(OH)_2D$), which has important effects on cell growth and differentiation. Laboratory studies have shown that 1,25 $(OH)_2$ D and its analogs inhibit cell proliferation and promote apoptosis in cell culture and animal models of breast cancer, causing delay in tumor development and regression of mammary tumors diagnosed previously[25].

Several polymorphisms of the gene encode the ligand VDR. One of them, identified by restriction enzyme Fokl produces RVD proteins differing in three amino acids. This polymorphism has been associated with increased risk of breast cancer in Afro-American women[10].

Other alterations that generate restrictions on BSML, ApaI and TaqI sites have been associated with alterations in receptor UTR region 3C is important in the control of post transcriptional gene expression. Polymorphisms in this region have also been linked to breast cancer; so that associations are confirmed between breast cancer risk and Apal polymorphism and progression of breast cancer and absence of TaqI genotype BSML with increased risk of metastasis[11].

In our study, we did not find any differences in the detection of the Fokl polymorphism among the two groups, nor did we monitor any prognosis in patients in which this mutation was shown. The number of patients with the mutation was limited and the possible conclusions obtained would not be reliable.

In 2010, the US Institute of Medicine defined as sufficient levels of vitamin D 20 ng/mL; deficiency considered concentrations 12 ng/mL or less and insufficient 12-19 ng/ml[26]. If we rely on these figures, none of our patients would have required any intervention and, as we found a correlation between levels of vitamin D equal to or less than 20 ng/mL with breast cancer. In this respect we agree with Manson et al. that this definition should be revised[27].

Bauer et al.[28] conducted a meta-analysis of prospective studies in which they analyzed the association and ranked them by menopausal status. They hypothesized that differences in condition and a non-linear dose-response relationship could be responsible for the discrepancies[1]. The meta-analysis included 9 prospective studies (published between 1996 and 2011) with 11 databases that evaluated circulating vitamin D levels in patients with breast cancer. 5,206 cases and 6,450 controls were included. Through a complex statistical analysis, they found a boundary relationship between circulating levels of vitamin D and breast cancer (RR for 5 ng/mL=0.99) in postmenopausal women, but not in premenopausal. They also found a flattening of the association at lower levels (27 ng/mL) or higher (35 ng/mL). The decreased risk in the range of 27-35 ng/mL in postmenopausal women was such that increments of 5 ng/mL in vitamin D levels were associated with decreased risk of breast cancer by 12% (RR=0,88 5 ng/mL).

With this study, the authors demonstrated the ability to determine an optimal range of vitamin D plasma levels (27-35 ng/mL) to decrease the risk of breast cancer in postmenopausal women. These findings should now be validated in studies incorporating individual-level data.

Another metanalysis evaluated a dose-response association[29]. This study included data from 15 publications that analyzed the relationship of breast cancer calcium intake in the diet. Seven studies

Table 1. Summary of results

Sun exposure less than 30 minutes a day				
	<30 min/day	>30 min/day		
Breast cancer	9	15	Prevalence in cases	0.63
Controls	15	37	Prevalence in controls	0.71
			Chi-squared=0.24	p=0.625
			Odds ratio	**1.48**
			CI 95%	**0.53-4.11**
			Population attributable risk	0.12
			Attributable risk in exposed	0.81
Deficient vitamin D intake				
	Deficient	Sufficient		
Breast cancer	18	6	Prevalence in cases	0.75
Controls	22	30	Prevalence in controls	0.42
			Chi-squared=5.79	p=0.016
			Odds ratio	**4.09**
			CI 95%	**1.04-11.9**
			Population attributable risk	2.81
			Attributable risk in exposed	3.33
Overweight/obesity				
	BMI >25	BMI 18-24.9		
Breast cancer	18	6	Prevalence in cases	0.75
Controls	21	31	Prevalence in controls	0.59
			Chi-squared=6.55	p=0.01
			Odds ratio	**4.42**
			CI 95%	**1.51-13.1**
			Population attributable risk	3.09
			Attributable risk in exposed	4.12
Very low levels of vitamin D				
	≤20 ng/mL	>20 ng/mL		
Breast cancer	18	6	Prevalence in cases	0.75
Controls	13	39	Prevalence in controls	0.25
			Chi-squared=14.99	p=<0.001
			Odds ratio	**9.0**
			CI 95%	**2.95-27.5**
			Population attributable risk	6.64
			Attributable risk in exposed	0.88

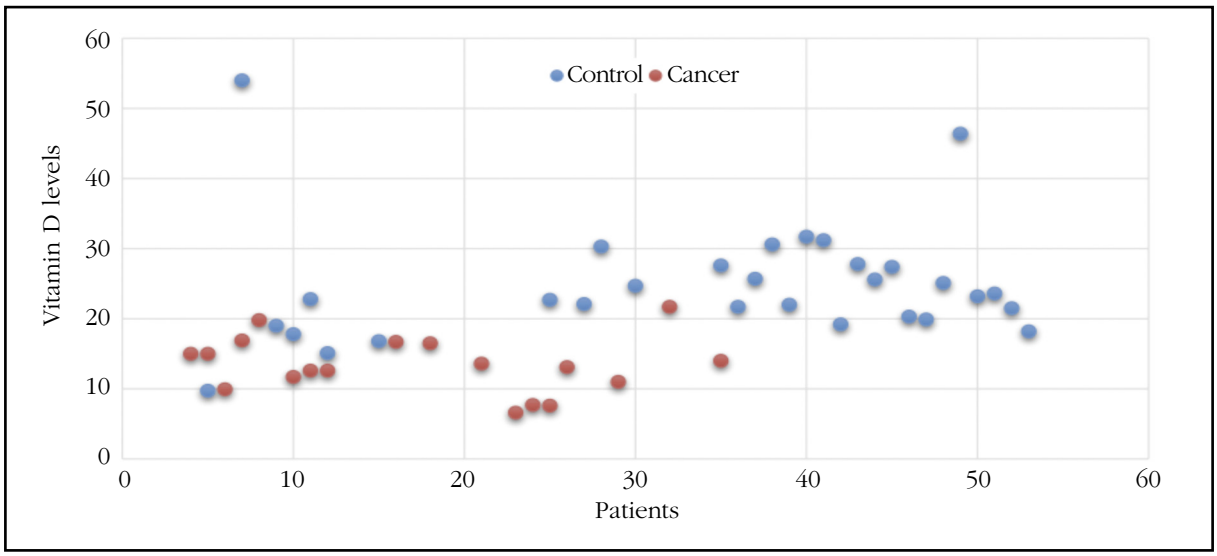
BMI: body mass index; 95% CI: confidence interval of 95%.

examined the association of breast cancer with serum levels of vitamin D and 11 reports where the relationship of this cancer with vitamin D intake were studied showed a linear association between calcium intake, a nonlinear relationship between vitamin D levels or vitamin D intake with the risk of breast cancer.

Chen et al.[29] suggest that women with a high intake of calcium, high vitamin D intake or those with adequate vitamin D serum levels present a lower risk of developing breast cancer.

Whether or not these data are conclusive concerning the association of low levels of vitamin D and breast cancer in all women, the benefits for

Figure 1. Levels of vitamin D in cases and controls



bone health and protection against other chronic diseases associated with adequate vitamin D levels are equally important. We must encourage patients to practice healthy lifestyle habits that help increase levels of vitamin D, such as maintaining healthy weight, avoid smoking, increase physical activity, and appreciate the importance of vitamin D supplements in adults who do not observe these changes in lifestyle.

Competing interests: The authors declare no conflicts of interest.

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Hemochromatosis and osteoporosis, in reference to 4 cases

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Summary
Although most people's osteoporotic conditions treated in clinical practice may be categorized in the postmenopausal osteoporosis group or related to aging, there are some osteoporosis cases linked to the development of some other disease or identifiable factor.
Most of these causes are associated with the taking of steroids, hypogonadism, malignant processes such as multiple myeloma, gastric surgery, alcoholism and treatment with anticonvulsant drugs. Hereditary hemochromatosis is another disorder related to the onset of osteoporosis. In this paper, we present 4 cases of patients with osteoporosis who also suffer hereditary hemochromatosis. The latter's characteristics are described and also its possible relationship with bone disease.

Key words: *hemochromatosis, osteoporosis, iron.*

Introduction

Osteoporosis (OP) is a disease characterized by decreased bone resistance with bone mass quality and quantity alteration that leads to disease fractures or fragility. The forearm, the vertebral bodies and the hip are the most common locations.

Risk factors include age, early menopause (and any case of low estrogen production), fractures due to previous personal fragility (this being the most important risk factor)[2] or in first-degree relatives, inadequate intake of dairy products, chronic gluco-corticoid intake (prednisone at doses ≥5 mg/d for 3 months), low body mass index (BMI) (<19 mg/m^2), high and chronic alcohol and caffeine, and smoking, as well as all those diseases that may cause a secondary OP (hypogonadism, hyperthyroidism, diabetes mellitus, renal failure and liver diseases, among others).

Hereditary hemochromatosis (HH), although uncommon, is one of the liver diseases described that can lead to the onset of OP. The most frequent form of presentation is related to the HFE gene (HH-HFE). Here we describe the existence of OP in 4 people suffering from HH-HFE.

Clinical Cases

At our hospital's metabolic bone disease center, 4 women were diagnosed with OP and as suffering from HH. We do not know the actual incidence of OP in patients with HH in our center as bone density DXA has not yet been carried out on all of these patients.

1st patient: She was diagnosed with HH-HFE and pituitary hypogonadism at 25 years and with OP at 51 years. Given the history of hypogonadism, hormone replacement therapy was started for amenorrhea and subsequently modified to raloxifene with supplements of calcium and vitamin D, with periodic follow-up. In the last review, DXA presented a T-score of -2.1 in the femoral neck (stable during treatment) and -3.2 in the lumbar spine (with slight deterioration since onset, since we started from -2,8). She did not present fractures during this time. When studying the risk factors of OP, it was observed that the patient presents adequate calcium intake, exercises regularly, is a non-smoker and has an adequate thyroid function. HH-HFE did not cause organic involvement, since it had been followed and controlled since its diagnosis, and 2 phlebotomies were performed during follow-up, with normalization of the analytical parameters of iron; she has always presented alkaline phosphatase within normal limits.

2nd patient: She was diagnosed with OP and HH-HFE (normal heterozygote/H63D) at age 64, although she had a history of vertebral fractures at age 55 and ribs at age 61 and 63 years. She has been treated for 4 years in our center, and the last DXA detected a T-score in the femoral neck of -2.6 (stable during follow-up) and in the lumbar spine of -2.1 (slight improvement with respect to initiation of treatment, -2.7). Initially she was treated with calcium and vitamin D. Subsequently, ibandronic acid was added and this was later replaced by denosumab until the present time, as the patient has not presented any new fractures. As for OP risk factors, she presents low calcium intake in the diet, is a smoker of 40 cigarettes/day and had menopause at 45 years. HH-HFE did not produce organic involvement since it was diagnosed and at no time did it require phlebotomies. In her follow up she has always presented alkaline phosphatase in the normal range. As a significant personal history, at 66 years the patient was diagnosed and surgically treated for colon carcinoma and, in addition, is a heterozygous carrier of the prothrombin mutation.

3rd patient: OP was diagnosed at 69 years of age and at 74 years of HH-HFE (heterozygote for C282Y) due to alterations in the ferric profile, which were already observed at the time of PB diagnosis. She was treated with calcium and vitamin D during the 5 year follow-up, and in the last DXA she presented a T-score at the femoral neck of -1.1 (worsening with respect to the time of diagnosis: 0.3, but slight improvement compared to the previous one: -1.5) and -2.3 in the lumbar spine (slight improvement compared to the start: -2.6). He did not suffer fractures during these years. As for her risk factors for OP, she has 2 children with whom she breastfeed and presented menopause at age 45, is an ex-smoker, has a low intake of foods rich in calcium, does not practice physical exercise routinely and does not have a history of fractures. HH-HFE is adequately controlled without organic alterations and at no time needed phlebotomies; During his follow-up he always presented alkaline phosphatase in the normal range. As an important pathological antecedent, he was diagnosed of chronic hepatitis C virus in response to antiviral treatment, which was maintained 9 years later.

4th patient: Diagnosed HH-HFE and later lumbar OP at 55 years. It has been followed in our consultation for 8 years, and in the last DXA presented a T-score at the femoral neck of -1.1 (being the initial of -1.6) and in the spine of -2.8 (maintained stable with respect to the start of treatment). At the beginning and during her evolution she was treated with calcium, vitamin D and raloxifene, without having presented fractures. As for her OP risk factors, she has 1 child and did not breastfeed, had menopause at age 51, is not a smoker, has adequate intake of calcium in the diet and exercises routinely. She has no previous history of fracture. She did not present organic alterations by HH-HFE and it was not necessary to practice phlebotomies during her follow-up. The alkaline phosphatase was always within the normal range. As an important personal antecedent, she was treated for chronic hepatitis C, with adequate response.

Discussion

Hepatic osteodystrophy refers to osseous diseases (mainly OP and osteomalacia, although the latter is very rare) secondary to chronic liver diseases[1,2,6,12], such as HH-HFE.

HH is a disease characterized by increased tissue deposits of iron secondary to decreased production or resistance to hepcidin, a hormone that in situations of excess iron decreases the intestinal absorption of iron by the enterocytes and the release of iron by macrophages[1,10]. This raises blood iron and its reserves in the organism with the consequent formation of pathological deposits in various tissues (liver, heart, pancreas, joints, bones, pituitary and skin, among others), generating multiple symptoms depending on their location. Most patients are asymptomatic in the early stages of the disease as they do not yet have these deposits. It should be noted that patients with HH present an increased incidence of cirrhosis and hepatocellular carcinoma[1,3,11].

HH is a predominant disease in the Caucasian population and 5 types have been described. The most common is inherited genetic alteration in an autosomal recessive form related to the HFE gene, the most severe presentation being homozygous C282Y, and the least relevant H63D[10]. It should be noted that the fact that a patient is homozygous for the C282Y mutation is not a diagnosis of HH if there is no elevation of the iron stores[1], since the HFE gene has quite a variable penetrance and it is not possible to ascertain which patients homozygous for C282Y will develop the disease[3].

Among the conditions related to iron overload, HH-HFE is the most common and the one in which most significant clinical complications occur, although we also find less common secondary causes, such as thalassemia major, sideroblastic anemia, multiple transfusions, long-term hemodialysis, chronic hepatitis B and C, alcoholic and non-alcoholic liver disease, among others.

The treatment for iron overload involves periodic phlebotomies until the normalization of the analytical parameters, which not only manage to control the levels of iron deposits in the body, but also improve and sometimes secondary complications of this disease disappear[1].

As for bone tissue, arthritis and OP have been found to be the main bone alterations related to HH-HFE, with arthritis being the most commonly associated, reaching up to 80% of patients. Treatment with phlebotomies does not manage to completely reverse this once it is already established[1,9]. The association between OP and HH has been known since 1960 and the incidence of OP is approximately 25-45%[5-7,9]. In a study conducted in Brazil, the presence of arthropathy, hepatocellular carcinoma, osteoporosis and diabetes was more common in HH-HFE patients compared to patients with iron overload from other causes[3].

The mechanisms by which OP occurs in chronic liver diseases are not fully known, but in HH-HFE it is thought that increased blood iron, not cirrhosis, is the main cause of this association, although in cirrhotic patients, involving a higher incidence of hypogonadism, it is known that the number of OP cases increases compared to patients without hypogonadism or cirrhosis[2,5,7,9]. Advanced HH may lead to cirrhosis (with or without hypogonadism) which adds to the deleterious effect of excess iron on the bone.

In 1989, Terrence Diamond et al. considered that excess serum iron altered the function of osteoblasts by decreasing osteoid matrix synthesis[7], which has been corroborated by two other studies, both *in vitro*, one of which concludes that elevation of serum iron decreases bone mineralization With increasing ferritin and its feroxidase activity, since it alters the function of osteoblasts by modifying the activity of the genes of CBF-α1 (involved in the maturation and differentiation of osteoblasts), osteocalcin and alkaline phosphatase in dose-dependent form[4]. The other study also concluded that iron overload produces OP by inhibiting the proliferation, differentiation and mineralization of osteoblasts, as well as decreased alkaline phosphatase activity[8].

This has also been studied by Valenti et al. who consider that OP in HH is related to hypogonadism, severity of iron overload and low weight, differing in alkaline phosphatase, since they found that high levels are also correlated with OP. This may be due to its relation to hypogonadism (in which bone resorption is increased, since it stimulates osteoclast activity)[5,7,12].

A suitable DXA diagnosis should be performed for all patients with chronic liver disease, including HH (especially HH-HFE)[2,5,12], although the timeframe determination for performance of this study in the follow-up of patients has not been determined[2,6]. The most common location of T-score decrease in DXA in patients with HH-HFE is the lumbar spine, followed by the femoral neck[5,9].

Regarding the treatment of OP, following the same guidelines is recommended as in patients without hepatic disease, adjusted according to individual characteristics. Avoiding hormone replacement therapy in severe liver disease is also recommended12. To reduce iron overload in HH-HFE patients, periodic phlebotomies have been shown to improve the ferric profile and thus improve osteoblastic function, which can sometimes be reflected in a decrease in the T-score value of the DXA[5,7,12].

As for the 4 patients presented here, HH-HFE was diagnosed based on analytical alterations at an early stage, and without any target organ damage in any of the cases. Taking into account that this diagnosis has preceded or been performed simultaneously with that of the OP (except for the 3rd patient, although the analytical alterations were already present at the time of OP diagnosis), we may consider it a risk factor associated with each patient's other clinical data and not as the main cause of OP, so that monitoring and analytical control have been performed to treat it and avoid complications of HH-HFE as would be done in any patient without OP.

In the first patient, hypogonadism is also an associated risk factor for presenting OP, although this pathology has always been controlled by the gynecology service since its diagnosis. The next patient has low calcium intake and is a smoker, which also

contributes to the presence of OP together with HH-HFE. Being a smoker, low intake of calcium-rich foods and lack of physical exercise are other risk factors associated with OP that the third patient presents. She also presented slight alterations of the ferric profile without needing phlebotomies to correct it. Finally, the last patient had no other associated factors except treated hepatitis C, although she did not develop cirrhosis or other complications.

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Oxidative stress as a possible therapeutic target for osteoporosis associated with aging

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Summary

Senile or involutional osteoporosis is a major problem in the developed world. Recent studies point to increased oxidative stress associated with aging, whether biological or chronological, as an important factor in its development. In this review paper, we focus on bone tissue disorders related to aging, the source of oxidative stress and negative influence on bone tissue. Finally, we consider the potential oxidative stress therapies currently being developed for this disease.

Key words: *oxidative stress, osteoporosis, aging, fragility.*

Introduction

The aging population in developed nations has led to an increase in the prevalence and incidence of osteoporosis. An estimated 200 million people suffer with this condition worldwide[1].

Defined as a decrease in bone mass and quality that increases the risk of fracture[2], osteoporosis is closely related to aging. Although the factors involved have not been fully identified, those associated with involutional osteoporosis include estrogen after menopause[3], glucocorticoid deficit therapy[4], diabetes mellitus (DM), primarily type 2[5]; renal failure[6] (which causes secondary hyperparathyroidism) and, more recently, increased oxidative stress associated with many of these conditions[7]. In this review paper, we consider the role of oxidative stress in bone metabolism as well as possible alternative drug therapy to mitigate harmful effects in cases of osteoporosis.

Bone disorders associated with aging

Bone tissue undergoes a continuous remodeling process, with considerable regenerative capacity and adaptation to physiological changes. This process takes place in so-called bone remodeling units, consisting of different cell types: osteoclasts, osteoblasts and osteocytes (fully differentiated osteoblasts embedded in the mineralized matrix and actual orchestrator of remodeling process)[8]. Bone remodeling is highly regulated by genetic, mechanical, hormonal and local factors which determine the outcome of bone balance.

Peak bone mass is reached during puberty in women and somewhat later in males. The latter group develop a higher bone mass and present larger, wider bones, while the female bone structure tend to be smaller in diameter and cortical thickness. From about 30 years of age, a negative bone balance is observed in both sexes (with a predominance of bone resorption) which leads to a gradual loss of bone mass similar in both sexes, initially in the trabecular bone and later in the cortical[3]. This decline is accelerated after menopause in women due to loss of estrogens, agents with proven antioxidant properties, which maintain lower bone mass than in the case of men during aging. With age, metabolic disorders that affect the bones occur: neuromuscular changes related to lack of mobility; increased endogenous glucocorticoid production and renal failure with decreased synthesis of calcitriol. Moreover, with aging, bone collagen fibers undergo structural changes and the bone loses the ability to repair microfractures[9]. All this contributes to the increased incidence of fractures.

Most current concepts on the development of senile osteoporosis have been obtained from studies in experimental models, mainly in rodents. However, when interpreting these results, some bone peculiarities in rodents compared to humans must be taken into account, such as continuous modeling bone from the growth plate, the absence of menopause, as well as a lack of Haversian cortical bone system. However, as in humans, rodents have shown bone mass loss and a deterioration of structure and of long bone regenerative capacity associated with aging[10,11]. The bone loss in aged rats is related to a decrease in osteoblast maturation and the increased number of osteoclasts compared to osteoblasts in the trabecular bone[12]. Also, in inbred mice in which bone mass is regulated primarily by genetic factors, bone loss associated with age may assume up to 10% of the total bone mass, which is attributed to decreased bone remodeling[13-16].

As observed in rodents, humans initially tend to lose trabecular bone with age, especially in women[17], related in part to a decrease in physical activity and, therefore, the mechanical stimuli in the tissue[18]. From 70 years, decreased cortical thickness is more pronounced with a concomitant increase in the intracortical porosity of the femur. The medullar area increases both in men and women[19]. These changes are associated with increased risk of osteoporotic fractures. However, in both mice and humans, the mechanical properties of bone are relatively conserved through a sustained increase in sub-periosteal mineral, which increases inertia time[20].

Mechanisms associated with bone aging

The underlying molecular mechanisms of involutional osteoporosis have begun to be elucidated in recent years. Associated with age, there has been a decrease in the osteoprotegerin (OPG) ratio/ligand receptor activator of nuclear factor (NF). This ratio is an important modulator of the remodeled bone[21]. Both OPG and RANKL are produced and secreted into the extracellular medium by osteoblastic cells and osteocytes. In fact, studies in mice models indicate that osteocytes produce most RANKL, thus directly influencing bone remodeling[22,23]. OPG is a soluble decoy receptor that captures RANKL in the extracellular medium (or on the surface of osteoblasts) and prevents it from binding to its receptor (RANK) in cells of osteoclastic lineage, thereby preventing the maturation and activation of osteoclasts. Thus, the OPG/RANKL relationship is an important anabolic/catabolic balance factor during bone remodeling[24]. Thus, the decreased OPG/RANKL relationship with age is consistent with increased osteoclast precursors in the bone marrow of old mice[25]. Osteocyte apoptosis plays an important role in bone loss associated with age and to immobilization or lack of stimuli[26-28] and also associated with an increased RANKL expression[21]. Moreover, in old mice of the C57BL/6 strain, an increase in the production of endogenous glucocorticoids has been observed through the activation of the enzyme 11 beta-hydroxysteroid dehydrogenase type 1. This is related to reduced viability of bone cells (osteoblasts and osteoclasts) and angiogenesis, a key process in bone formation[29].

Several factors may affect the rate of fracture repair with age[30]. With aging, there is a decrease in bone marrow osteoprogenitor, which occurs in parallel with increased adipognesis[31]. Both osteoblasts and adipocytes share a mesenchymal precursor cell differentiable either lineage depending on the microenvironment which are exposed these cells. Furthermore, osteoblasts from old

mice RANKL production increase parallel to the decrease in expression of OPG. This alteration results in increased osteoclastogenesis and osteoclast activity[21,25]. It is noteworthy that there are a decreased number of endothelial cells and angiogenesis, which may contribute negatively to the process of bone repair in older people[32].

Recently an increase in bone mass and reduced risk of fractures have been observed in elderly subjects who undergo angiotensin II receptor antagonist treatment[33]. The drug's apparent beneficial effect on the bone is attributed to the inhibitory action of angiotensin II on various osteoblast differentiation markers, such as runt-related transcription factor 2 (Runx2), essential for osteoblast differentiation, osteocalcin[34] and the increase of RANKL, which favors osteoclast differentiation[35]. These data suggest that high blood pressure which is prevalent in the elderly could also contribute to involutional osteoporosis.

Sclerostin, the osteocyte-derived product of the Sost gene, is a potent inhibitor of bone formation through the binding to receptors associated with low density lipoprotein 5 and 6, inhibiting the canonical Wnt. Recent studies have shown that circulating sclerostin increases in post-menopausal women and with age in both sexes, which could have a negative influence on bone mass[36,37].

Currently, the product of the klotho gene is known to be an important modulator of cellular aging[38], a transmembrane protein acting as fibroblast growth factor (FGF) co-receptor 23 produced by the osteocytes and inducer of phosphaturia. Mice deficient in the Klotho gene suffer accelerated aging and osteopenia characterized by a decrease (20-40%) of cortical thickness in the femur, tibia and vertebrae, and low bone remodeling with a very sharp decline in cortical bone formation. Stromal cells from the bone marrow of these mice have a reduced ability mineralized nodule formation and phosphatase alkaline activity[39]. Paradoxically, these Klotho deficient mice have increased trabecular bone in the vertebrae and the metaphysis of long bones; an effect which the authors attribute to a selective activation of the Wnt pathway on the trabecular component. Klotho interacts with the Wnt pathway through its secreted product, which binds to ligands of this pathway by inhibiting its action, hence the absence of Klotho could lead to activation of the pathway Wnt[39]. Furthermore, mice without telomerase have been shown to exhibit increased cellular senescence and a decrease in bone mass 3 months from birth, associated with a reduction in bone formation and osteoblastogenesis[40]. Apparently, this reduction is because mice without telomerase have poorly differentiated osteoblasts and the pro-inflammatory environment that promotes osteoclast activity.

Oxidative stress as a pathogenic factor in involutional osteoporosis

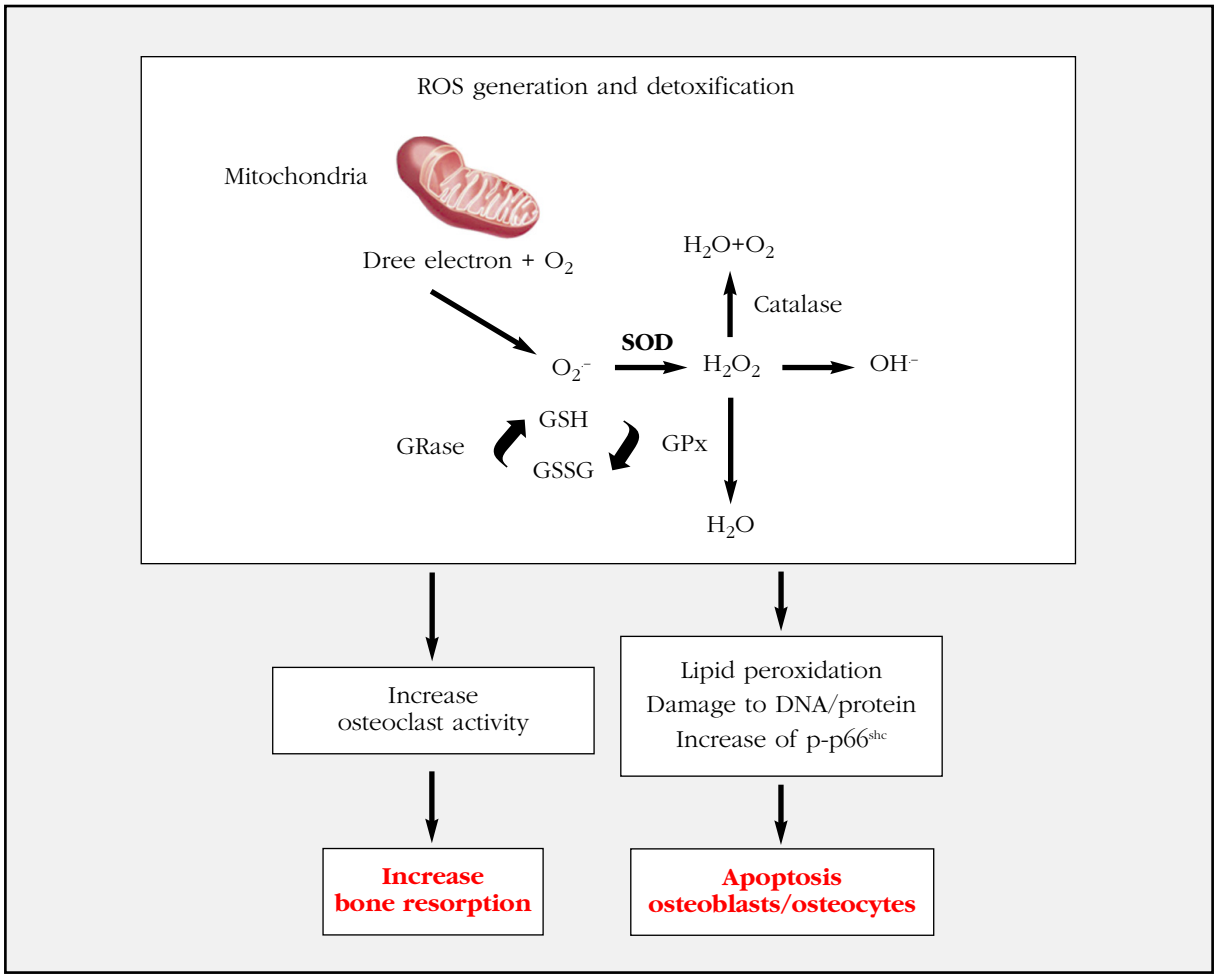
Aging can be seen as a consequence of the imbalance between oxidizing agents produced naturally in cell metabolism and antioxidant defenses, with a predominance of the first. This is known as oxidative stress, which involves the oxidation of biomolecules and functional loss of cells[41,42]. Increased oxidative stress, carried out primarily in the mitochondria, is based on the overproduction of reactive oxygen species (ROS) such as superoxide anion (O_2^{-}), hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2).

This increase cannot be properly balanced by antioxidants systems such as superoxide dismutase (SOD), catalase (CAT) enzymes glutathione cycle (glutathione reductase and glutathione peroxidase) and thioredoxin, among others. Excess ROS with chronological (and/or biological) age oxidizes DNA, proteins and lipids and induces the phosphorylation of mitochondria $p66^{shc}$ protein, leading to cell death[7,43-45] (Figure 1). Recently, oxidative stress has been found to have important functions in cell signaling[46,47]. In this context, ROS can be considered second messengers of inflammatory response. In fact, oxidation and inflammation are two closely related processes that increase with age[48].

Although some researchers have raised questions about whether oxidative stress is a cause or consequence of aging, in recent years it has been implicated in the bone deterioration[49]. Using various animal models: premature aging, osteoporosis due to estrogen deficit (after ovariectomy) or diabetes, increased oxidative stress markers was found to decrease bone formation mechanisms[50-54]. The effects of oxidative stress to induce deleterious effects on bone tissue are not yet well known. Increased ROS may stabilize forkhead box O (FoxO) transcription, an important family of transcription regulators of many genes. Its functions include control of glucose metabolism, tumorigenesis and cell defense against oxidative stress[55]. FoxO 1 and 3 are expressed in the bone 56, where they seem to play a key role in maintaining bone formation[56]. It has been shown that genetic deletion of FoxOs in mice increases oxidative stress in bone and induces bone loss trabecular and cortical, associated with increased osteoblast/osteocytic apoptosis and a decrease bone formation[57]. The activation involves FoxO phosphorylation engagement with the beta-catenin[57] causing gene induction of oxidative stress response, as GADD45 and CAT[58]. In fact, the protective action of oxidative stress of Klotho protein appears aforementioned mediated activation FoxOs[39]. Furthermore, activation of the FoxO prevents beta-catenin to act as transcription factor in stimulating the proliferation and differentiation of osteoblasts[56].

Increased ROS in bone cells causes damage and apoptosis genomic DNA of osteoblasts and osteocytes. In addition, lipid peroxidation dependent lipoxygenase activated by oxidative stress plays an important role in bone loss associated with aging. This is evidenced by analyzing the expression of the lipoxygenase and ALOX12 and formation Alox15 4-hydroxynonenal, a product of lipid peroxidation, increased bone in older mice[59]. It has also been shown that products of lipid oxidation inhibiting action osteogenic factors[60].

Figure 1. Generation and cell damage caused by excess ROS. ROS generation is a consequence of aerobic metabolism in the mitochondrial respiratory chain. Enzymes such as SOD, glutathione reductase CAT and/glutathione peroxidase system are responsible for maintaining physiological levels of ROS. However, when this balance decompensates excess ROS synthesis, cell damage may occur leading to apoptosis of osteocytes and osteoblasts and increased osteoclast activity



Furthermore, the increase of ROS has been linked to an increase of osteoclastogenesis and osteoclast activity[61,62].

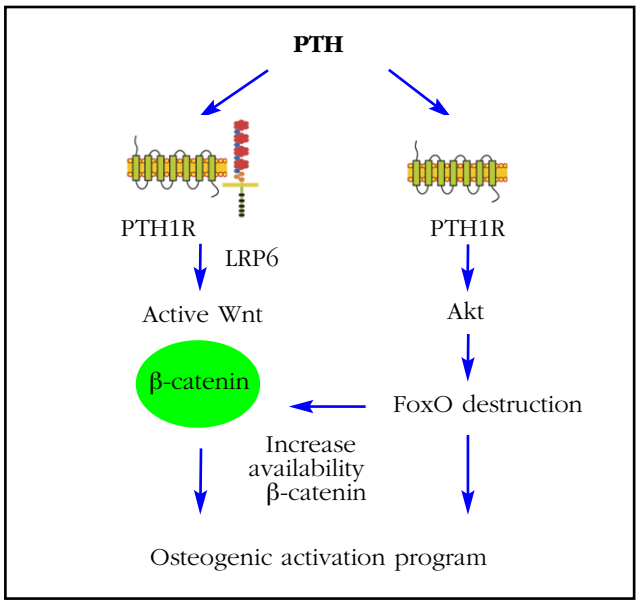
It has recently been shown that the enzyme nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX 4) plays a key role in osteoclastogenesis. Mice deficient of this enzyme, which produces constitutively ROS have a high bone mass and osteoclast markers deficit; also in human bone samples high osteoclast activity is correlated with increased activity of NOX 4[63]. Furthermore, it is noted that in situations of increased ROS associated with experimental DM, are mixed results. While some authors have observed an increase in osteoclast activity[64], it has been suggested that could be related to the greater severity of DM[65], however, other DM models, osteoclastic activity is reduced[66]. In fact, studies using murine osteoclasts pre-incubated in the presence of high glucose appear to confirm their inhibitory effect on osteoclasts[67]. Thus, differences in the degree of DM, strain and age of the animal, could contribute to the varying levels of bone resorption observed in different models[65,68].

Possible oxidative stress therapies in senile osteoporosis

The development of new anabolic therapies for osteoporosis that combine increased bone mass with its ability to neutralize the harmful effects of oxidative stress is of great interest. An intuitive approach to prevent bone loss with age would be based on the antioxidant administration. However, it pointed out that classic antioxidants, such as the CAT or N-acetylcysteine, exert undesirable effects on bone tissue as authentic anti-osteoclastogenic act as agents interfering with bone remodeling[69]. In addition, such agents inhibit the canonical Wnt/beta-catenin whose activation is vitally important for maintaining bone formation, partly by inducing the seizure of activating the protein disheveled by the regulatory protein redox balance, nucleoredoxin[70].

Recently, the bone anabolic effect has been associated with intermittent administration of parathyroid hormone (PTH) with its stress oxidative properties, such as the decrease in the amount of ROS, inhibition of phosphorylation of p66shc adaptor protein and increasing the amount of total glutathione[69]. The advantage of this treatment with PTH versus the clas-

Figure 2. Osteogenic actions of PTH through the Wnt/beta-catenin pathway. PTH is able to directly activate the Wnt pathway by binding of the type 1 receptor (PTH1R) with coreceptor Wnt proteins, the related receptor low-density lipoprotein 6 (LRP6). Furthermore, phosphorylation of Akt activation produced by PTH1R results in FoxO degradation, which favors beta-catenin stabilization



sic antioxidants determines its stimulatory action of bone remodeling, with a predominance of bone formation in part through its interaction with the Wnt/beta-catenin (Figure 2). In this context, *in vitro* testing has been shown that the N-terminal (1-36) (homologous with PTH) and C-terminal (107-109) of the PTH-related protein (PTHrP) fragments are able to counteract oxidative stress induced by H_2O_2 in osteoprogenitor cells relative to their osteogenic action[52,71].

In vitro studies and animal models suggest that resveratrol, a compound bifenilic group of polyphenolic antioxidants present in the skin of grapes and other fruits[72,73], could be a potential anti-osteoporotic agent. This compound increases the proliferation and differentiation of osteoblast in the pre-MC3T3-E1 mouse *in vitro*[73]. Furthermore, administering resveratrol to mesenchymal cells derived from human embryonic stem cells has been shown to induce the expression of mature Runx2[74] differentiation[75] and osteoblasts. This mechanism of action of resveratrol appears to be mediated by SIRT1 deacetylation activation which increases FoxO3a expression and complex formation with resveratrol, increasing Runx2 expression (Figure 3). SIRT1 could also increase the activity of Runx2 directly by deacetylation of this transcription factor in pre-osteoblast cells. In recent research into older rats, administering resveratrol (10 mg/kg daily for 10 weeks) has been shown to improve bone quality and bone biomechanical properties of the osteoporotic bone[76]. Although these pre-clinical results are promising, there are still no hard data to confirm the efficacy of resveratrol in senile osteoporosis in humans. However, of note is a recent study conducted in obese and osteopenic patients, in which oral administration of resveratrol (1 g daily for 16 weeks) significantly increased bone mass, and the amount of bone alkaline phosphatase, compared to the placebo group[77]. Recent reports indicate that mice deficient in SIRT6, another deacetylase related to the response to oxidative stress, present an osteoporotic phenotype at an early age. The absence of SIRT6 is associated with overexpression of Runx2, osterix and OPG as well as the increased Wnt pathway inhibitor, Dickkopf 1, which leads to a deficit of osteoblast and osteoclast maturation[78]. These data suggest that SIRT6 could be a therapeutic target in involutional osteoporosis.

Furthermore, glucocorticoid excess also induces oxidative stress. In this situation, the oxidative stress observed in plasma reticulum can be reversed by translation initiation factor 2α phosphorylation, which disrupts protein translation. A dephosphorylation inhibitor compound, salubrinal, has recently been shown to prevent deficit mineralization of osteoblasts treated with glucocorticoids *in vitro* as well as osteoblast and osteocyte apoptosis in an osteoporotic mouse model by prednisolone administration[79].

Conclusions

The progressive aging of the population in the developed world leads to increased musculoskeletal disorders, including osteoporosis. Osteoporosis and increased fragility of the elderly population are a socioeconomic challenge of the first magnitude. Different factors contribute to bone loss in the elderly, among which stands out as a common element increased oxidative stress (Figure 4). Thus, reducing oxidative stress could be a useful tool to combat involutional osteoporosis. However, the fact that oxidative stress compounds could interfere with the bone remodeling or key anabolic pathways for bone formation, such as the Wnt signaling pathway, requires certain considerations prior to therapeutic use. We must also take into account the physiological role of ROS, which act as secondary messengers of many metabolic pathways; therefore its uncontrolled inhibition could lead to unwanted side effects in bone cells. Further research is needed to determine the true effect of antioxidant therapies and appropriate dosing schedules to avoid deleterious action on bone remodeling. Taking into account these considerations, therapies aimed at neutralizing oxidative stress to prevent or alter the course of involutional osteoporosis would represent an obvious medical breakthrough.

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Figure 3. Osteogenic action of the resveratrol by interaction with SirT1. Resveratrol induces increased osteogenic factor activity by Runx2 deacetylation transcription mediated SirT1 deacetylase. It also promotes the formation of a transcription complex between FoxO3a and SIRT1 that promotes increased Runx2 expression

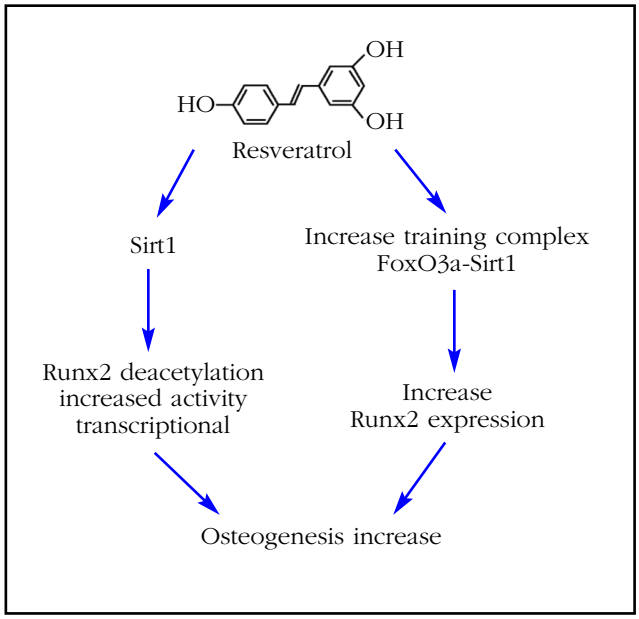
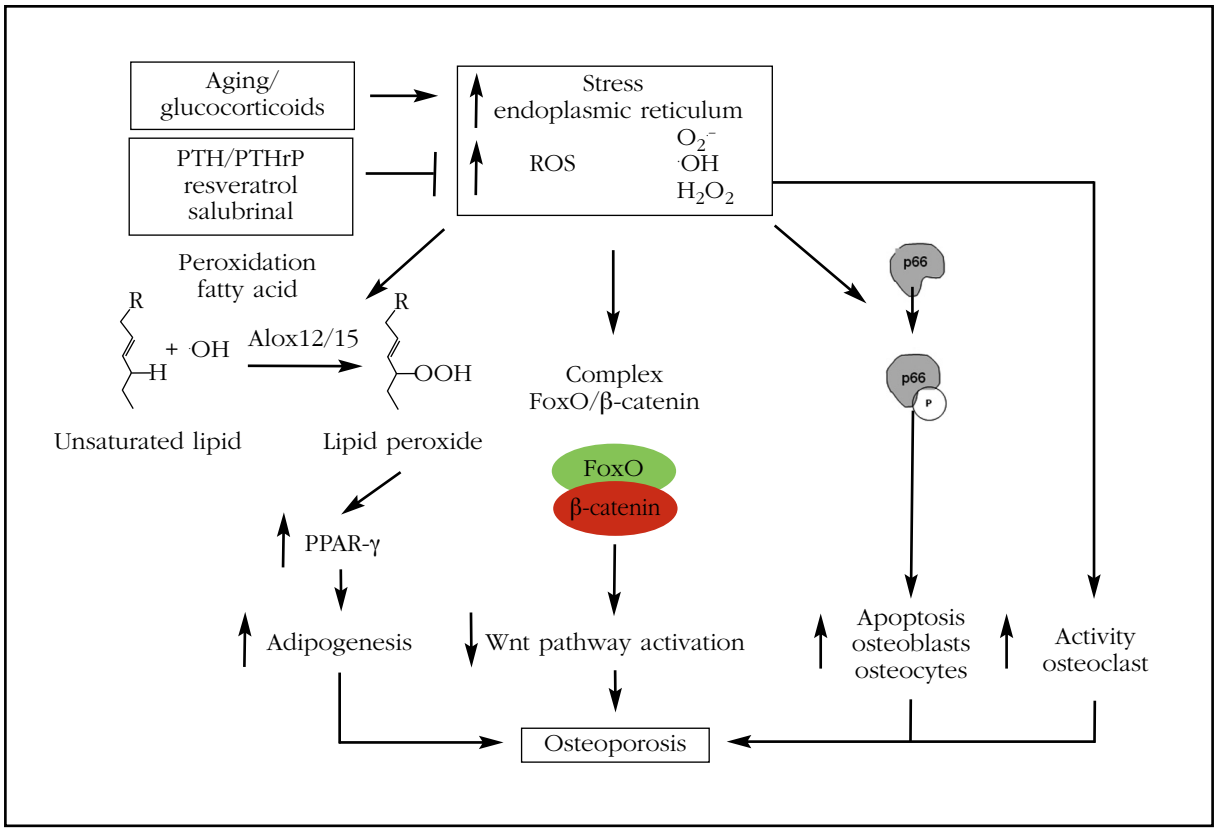


Figure 4. Role of oxidative stress in bone damage associated with aging. Alteration of mitochondrial homeostasis with age causes generation of excessive amounts of ROS that exceed the capacity of cellular detoxification systems. Bone-forming cells in, excess ROS results: an increase in receptor expression activated gamma peroxisome proliferator (PPAR-gamma3); FoxO coupling with PPAR beta-catenin, which inhibits the Wnt pathway; and p66 protein phosphorylation inducing apoptosis. This excess ROS favors increased osteoclast activity and osteoclastogenesis. Together these facts altered bone remodeling. The oxidative stress activity of agents such as PTH (and PTHrP), resveratrol and salubrinal are associated with osteogenic actions



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