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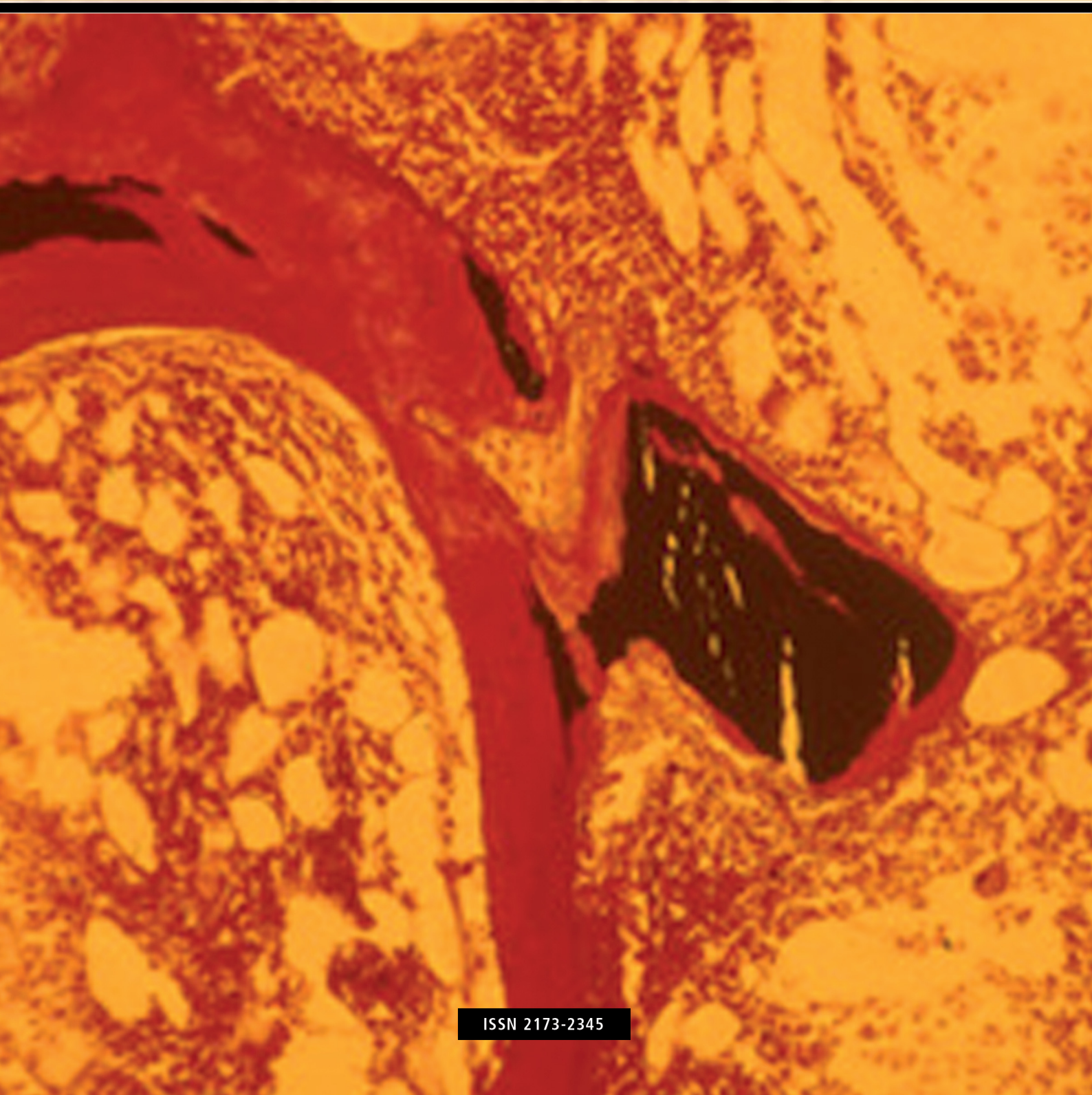

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Our cover: Patient with osteomalacia due to vitamin D deficiency (Von Kossa stain).
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Summary

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Bone protection during breast cancer treatment

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Few medical areas have changed as much through the last decades as the treatment of breast cancer (BC). From Halsted's theory of the progression of an initially local disease, with a first loco-regional and then metastatic extension, to the most recent studies in molecular biology that identify the gene personality of each tumor, there have been many advances. Old TNM classification originally designed for solid tumors have been abandoned and all areas related to hormonal dependence and gene expression of each tumor have grown in importance. All this is aimed at better facing a global therapeutic approach.

Almost 20 years ago, an important biological research laboratory provided us with a detailed study of the basal estradiol levels of the patients in the placebo group of the MORE study[1]. An increased risk of breast cancer associated with raised serum estradiol levels was demonstrated, confirming the previous results on the hormonal dependence of this neoplasm. With the introduction of chemotherapy (QMT) in the final decade of the last century, the general mortality of women from breast cancer was reduced in all western countries. At the time, and just a few years later, the implementation of massive early detection programs at the population level facilitated an increase in the diagnosis of tumors in early stages.

Currently, women survive BC for many years more than just twenty years ago, increasing the risk of various chronic diseases, to which little or no attention was previously given by oncology teams. To this we must add that treatments that seek to eliminate hormonal influence such as surgical oophorectomy, GnRH agonists, and QMT with consecutive induction of iatrogenic early ovarian failure, may increase the risk of loss of bone mass and the appearance of osteoporosis (OP) in surviving women.

Breast cancer *per se* does not influence the increased risk of OP. In fact, the prevalence of fractures among patients diagnosed with untreated breast cancer and who do not have bone metastases the frequency is similar to that of the general population[2]. In these women, the bone mineral density (BMD) in the lumbar spine, hip and radius is similar to that of healthy women. These results are observed in both premenopausal and postmenopausal women[3]. Significant changes in the biochemical markers of bone remodeling (BMBR) have not been reported in women with BC, at least before starting anti-tumor treatment[4]. So it does not appear that the prevalence of OP in women with BC is increased at the disease onset. At the same time, once again using the placebo groups of the trials as biological laboratories, it has been described that the proportion of patients with at least one event related to the skeleton is significantly higher in the group with BC than in the cancer patients generally related to bone damage, such as multiple myeloma or even prostate cancer[5].

The constant reduction in mortality from breast cancer, the diagnosis earlier and the increasingly selective but high intensity aggressiveness in the therapeutic approach, put on the table of the clinicians involved a new challenge: to avoid damage in these patients bone as a tribute that too many times, too many women pay to achieve a survival that, let us not forget, we are in a position to improve with an adequate quality of life.

Thus, anti-neoplastic therapy makes the difference in surviving patients with BC, regarding their bone risk. Premenopausal women with BC who receive ovarian irradiation also have accelerated bone loss as a result of cessation of ovarian activity. Regarding systemic treatment, both cytotoxic drugs and anti-hormonal therapies can facilitate the development of osteoporosis. The former, cytotoxic agents, in addition to acting on neoplastic cells, can alter osteoblastic and gonadal activity. The main cause of this disorder is cyclophosphamide, which, along with other drugs (methotrexate, doxorubicin, and fluoracil), is included in classic

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therapeutic regimens, all of which are capable of damaging the cells of the granular layer of the ovary. Gonadal dysfunction, which is present in most women at the end of treatment with this drug, can persist indefinitely depending on the age of the patient and the dose and duration of treatment[6]. Furthermore, regardless of the duration or dose of therapy, when ovarian failure occurs, patients develop a state of estrogenic deficiency and a subsequent increase in bone resorption[6]. This increase in resorption causes a decrease in BMD in the first years after the cessation of menstruation, decreasing vertebral bone density by 21% compared to eumenorrheic women of the same age. QMT effects on gonadal function seem to be responsible for the loss of bone mass that is observed in premenopausal women with BC who undergo QMT and that can exceed 5% per year.

By verifying the influence of QMT on fracture risk, it has been found that it is four times higher for vertebral fracture[7]. The data provided by one of the branches of the WHI (Women's Health Initiative) showed that the risk of presenting a vertebral or wrist clinical fracture is increased by 30% in postmenopausal women who have survived BC, while it does not appear that the incidence of hip fracture increases significantly[8]. Other authors also found inconclusive results for hip fracture[9].

The true workhorse in the past two decades has been the use of universal anti-hormonal therapies in patients with positive hormone receptor (HR) BC. The aromatase enzyme is known to be responsible for the peripheral conversion of androstendione and testosterone to oestrone and estradiol. It is present in the breast, fat, muscle and brain tumor tissue. The biological action of aromatase inhibitors (AI) is to block aromatase, inhibiting the cytochrome P450 isoenzyme, responsible for the peripheral conversion of androgens to estrogens. Estrogens maintain bone mass, and AI treatment involves rapid bone loss due to estrogen deficiency. Given that the main source of estrogens in postmenopause is extraovarian, the suppression of circulating estrogens is profound in these patients, approximately 95-98%. Thus, their indication is limited to postmenopausal patients. Third generation aromatase inhibitors are divided into two groups: steroidal or type I inactivators and non-steroidal or type II inhibitors. Exemestane, a steroid inhibitor and an andrendrendione analog, irreversibly binds the aromatase enzyme, while letrozole and anastrozole, type II inhibitors, reversibly bind the enzyme. Various *in vivo* animal studies suggest that exemestane (steroid) may be less detrimental to bone health than non-steroidal inhibitors, perhaps because it is structurally related to androstendione and has an affinity for the androgenic receptor. Its main metabolite in humans and rats, 17-hydroxyexamestane, is also androgenic and strongly binds to the receptor. By contrast, non-steroids have no proven androgenic effects[10].

All clinical trials have shown that its use always improves the disease-free survival period, and at the same time reduces the risk of contralateral BC (the existence of a BC being the main risk factor for the development of a second BC in the same woman).

However, AIs are able to significantly reduce the BMD of treated patients. In a sub-study of the five-year Arimidex trial, tamoxifen (TAM), alone or in combination (ATAC), postmenopausal women with MC and anastrozole therapy were found to have increased bone loss in the lumbar spine (LS) and total hip (TH), 6 and 7.2%, respectively, compared to those assigned to TAM (increase of 2.8 and 0.74%, respectively)[11]. In a substudy (206 patients) of the Intergroup Exemestane Study (IES), in which postmenopausal women who had taken TAM for two or three years were randomly assigned to switch to exemestane or to continue TAM, it was found that those who switched to exemestane experienced a greater decrease in BMD in LS (2.7%) and hip (1.4%) after six months, compared to those who remained with TAM (without changes in any of the places)[12]. Bone loss slowed in the remaining 18 months of the study, decreasing an additional 1 and 0.8% in LS and TH, respectively, in subjects assigned to exemestane.

In premenopausal women, in whom the main source of estrogen is the ovaries, AIs alone are not effective. However, in combination with gonadotropin-releasing hormone (GnRH) agonists, goserelin, AIs cause more bone loss than TAM. In the Austrian trial of the Austrian Breast and Colorectal Cancer Study Group (ABCSG)[13], premenopausal women were randomly assigned to TAM plus goserelin versus anastrozole plus goserelin. Half of each group received zoledronic acid (ZOL). Significant bone loss occurred in the subset of patients who did not receive ZOL (reductions of 17.3 and 11.6% in patients who received anastrozole-goserelin and TAM-goserelin, respectively).

Regarding BMBRs, in several of the previously described assays, both bone resorption (urinary n-telopeptide and serum C-telopeptide [CTX]) and training (serum bone-specific alkaline phosphatase [BALP], N-terminal propeptide 1 procollagen [P1NP]) increased significantly with AI treatment[11-13].

Whatever the case, the most important bone damage in BC patients on AI treatment is the increased relative risk (RR) of fractures. These reportedly appear in patients of age ranges much earlier than that observed in the general population, as early as age 50, involving even hip fractures[14]. Compared to TAM, all AIs significantly increased the RR of fractures: anastrazole 43% higher than TAM in one study[15] and 100% in another[16]; letrozole 48% in one study[17], 15% in another[18]; exemestane 45%[19].

In this issue, the first results of a large cohort in our country of patients with BC treated with AI are published, and these extremes of bone risk are verified[20]. In this cohort of almost 1,000 patients followed consecutively for up to five years and one after the end of their therapy, the authors observed that the main risk factor detected for incident fracture in patients treated with AI is the diagnosis of osteopenia or osteoporosis. In their hands, the FRAX® calculation and the determination of β-CTX levels were useful in identifying high-risk patients.

Indeed, a complete evaluation of mineral metabolism (with measurement of BMD, RX of CL and of the thoracic spine, as well as MBRO and quantification of 25 OH vitamin D, at least) must be unequivocally part of the diagnostic study of any BC in a pre-patient or postmenopausal. The bone risk inherent in anti-neoplastic therapies used as part of health care after initial surgery, either QMT or with various anti-hormonal therapies, particularly with AI, is frequently updated in very notable loss of BMD in all locations with increased RR of fractures at ages sometimes up to ten to twenty years earlier than would be expected from the usual development of osteoporosis.

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The constant reduction in mortality from BC, the diagnosis earlier and the increasingly selective but high intensity aggressiveness in the therapeutic approach, put on the table of the clinicians involved a new challenge: to avoid damage in these patients bone as a tribute that too many times, too many women pay to achieve a survival that, let us not forget, we are in a position to improve with an adequate quality of life. In this endeavor, the multidisciplinary care that includes the gynecologist with the oncologist and bone metabolism specialists (endocrinologists, rheumatologists and internists) depending on the place, is an objective that all centers that care for people with BC should consider more sooner than later. It is a challenge that we all must face.

Conflict of interests: Authors declare no conflict of interests.

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Risk factors for incident fracture in patients with breast cancer treated with aromatase inhibitors: B-ABLE cohort

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Summary

Objetive: Aromatase inhibitors (AI) have been associated with an accelerated loss of bone mass and an increased risk of osteoporosis fractures. This study assesses the risk factors for incident fracture in breast cancer patients receiving AI.

Material and methods: Prospective-observational cohort study of women with breast cancer who begin treatment with AI (B-ABLE cohort). Patients were treated for 5 years or 2 or 3 years if they had previously received tamoxifen. Bone health was assessed from the beginning of the treatment until one year post treatment by bone densitometry, bone remodeling markers, vitamin D levels and an anteroposterior and lateral spine radiography. The fracture risk calculation was performed using the FRAX® tool before starting AI. Cox models were used to calculate the risk ratios (HR [95% CI]) of fracture.

Results: A total of 943 patients were included in the study. 5.4% suffered an incident fracture, most during AI treatment, although 21.5% occurred during the first year after the end of therapy. Most of the incident fractures were clinical vertebral (29.4%) and Colles (31.4%). 86.3% of the patients had a diagnosis of osteopenia or osteoporosis at the time of the fracture and 33% had the levels of β-CTX (β isomer of the carboxyterminal telopeptide of type I collagen) above normal.
Patients diagnosed with osteoporosis or at risk of fracture at the start of the study were treated with bone antiresorptives. No significant differences in fracture risk were found between patients with and without antiresorptive therapy: HR=1.75 [95% CI: 0.88 to 3.46]. Nor were differences found among patients who had previously treated with tamoxifen compared to those who did not (HR=1.00 [95% CI 0.39 to 2.56]). The FRAX® tool gave average values within the intermediate risk range, with 13 patients with high risk of major fracture values.

Conclusions: The main risk factor detected for incident fracture in patients treated with AI is the diagnosis of osteopenia or osteoporosis. The calculation of the FRAX® tool and the determination of β-CTX levels are useful tools to identify high-risk patients.

Key words: aromatase inhibitors, fracture, breast cancer.

Introduction

Currently, aromatase inhibitors (AI) are used as first-line adjuvant therapy for women diagnosed with breast cancer with positive hormonal receptors. Although its effectiveness in reducing the risk of recurrence and mortality is well known[1], AIs have also been associated with side effects that can negatively affect the patient's quality of life, adherence to treatment and associated mortality[2].

In AI treatment, there is a marked reduction in circulating estrogens in postmenopausal women by blocking the conversion by the enzyme aromatase from androgens to estrogens. This action leaves the woman without residual estrogens, such as estradiol and estrone, after menopause. One of the most common side effects is accelerated bone loss, which is associated with an increased risk of osteoporotic fractures[3,4]. Along these lines, there are different meta-analyzes that include randomized controlled clinical trials that have shown an association between prolonged treatment with AI and an increased risk of bone fractures, with an increase between 34% and 59%[5,6].

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Furthermore, in a cohort study that included 1,775 patients who started long-term AI therapy, the risk of osteoporotic fracture was similar to that of the general population. It should be noted that in this study, AI-treated women presented a higher baseline BMI, a higher bone mineral density and a lower prevalence of fracture prior to the start of the study than the general population[7].

The B-ABLE cohort (Barcelona–Aromatase induced Bone Loss in Early breast cancer) includes postmenopausal patients with estrogen receptor-positive breast cancer (RE+), recruited at the time of starting AI treatment. This cohort has been used to conduct a prospective observational study in which patients are monitored throughout the study with bone health data and associated factors from the start of treatment until one year after the end of treatment[3].

This study was aimed at assessing clinical fracture incidence and the characteristics of patient fractures in the B-ABLE cohort during AI regime and one-year post treatment.

Material and methods

Study group

A prospective, unselected, observational and clinical cohort study was carried out in the B-ABLE cohort that included postmenopausal patients diagnosed with positive estrogen receptor (RE+) breast cancer, treated at the Hospital del Mar in Barcelona. Participants were recruited at the beginning of AI treatment (letrozole, exemestane or anastrozole) and were treated for 5 years, according to the American Society of Clinical Oncology recommendations, starting within 6 weeks post op or 1 month after the last cycle of chemotherapy[8]. Alternatively, those patients who were pre-menopausal at the time of starting adjuvant treatment were treated with tamoxifen for 2 or 3 years, and were included in the study at the time of changing to AI due to the onset of menopause. These patients were treated with AI (3 or 2 years, respectively) until completing 5 years of adjuvant therapy. In addition, all participants received calcium and 25(OH) vitamin D3 supplements (1,000 mg and 800 IU daily, respectively), and those with vitamin D deficiency (<30 ng/ml) received an additional dose of 16,000 IU of oral calcifediol or 25,000 IU of oral cholecalciferol every 2 weeks. Patients diagnosed with osteoporosis by bone densitometry (dual energy radiological absorptiometry, DXA), fragility fractures before starting AI, and/or a bone mineral density (BMD) with a T-score <-2.0 plus a factor of increased risk for osteoporosis, they started treatment with oral bisphosphonates or denosumab in the case of digestive intolerance or previous gastroesophageal disease. The patients maintained this treatment throughout the study.

Exclusion criteria was: alcohol addiction, renal failure > grade 3b, rheumatoid arthritis, bone metabolic diseases other than osteoporosis, Paget's disease, osteomalacia, primary hyperparathyroidism, hyperthyroidism, insulin-dependent diabetes mellitus, prior or ongoing treatment with antiresorptives, oral corticosteroids or any other drug that could affect bone metabolism, except tamoxifen.

The study protocol was approved by the ethics committee of the Parc de Salut Mar (2016/6803/I) and was carried out in accordance with the Declaration of Helsinki. Written informed consent forms were obtained from all participants after reading the study information sheet and answering any questions. Patient privacy rights were respected at all times.

Data and patient measurements

Information on clinical and demographic variables was collected at the time of recruitment and during the study, including age, menarche and menopausal age, body mass index (BMI), diet and lifestyle, chemotherapy and previous radiotherapy, tamoxifen previous, antiresorptive treatments, family history, previous falls, serum levels of 25(OH) vitamin D (VitD) and paratohormone (PTH), as well as the following parameters of bone remodeling: aminoterminal propeptide of type I collagen (P1NP), the isomer beta of the carboxyterminal telopeptide of collagen type I (β-CTX), osteocalcin and bone alkaline phosphatase. Before the start and annually until after one year after the end of the AI treatment, bone mineral density (BMD) was measured at the lumbar level (CL L1–L4), femoral neck (CF) and total hip (CT), using the DXA QDR 4500 SL® densitometer (Hologic, Waltham, Massachusetts, USA). The coefficient of variation for this technique in our center is 1% in CL and 1.65% in CF. Those images that presented degenerative disc disease with osteophytes, osteoarthritis with hyperostosis of the facet joints, vertebral fractures and/or aortic calcifications and all those that could cause a false increase in BMD were excluded, according to the follow-up. description of Blake et al.[9]. Incident fractures were diagnosed by a lateral x-ray (Rx) of the dorsal and lumbar spine by a specialized doctor or by a medical report from another center. The risk of fracture at 10 years was assessed using the FRAX® tool on the platform, with access at: https://www.sheffield.ac.uk/FRAX/tool.aspx?lang=sp. The thresholds of FRAX values that were used to identify people with high or low risk of main osteoporotic fracture in the Spanish female population were: low risk, <5; intermediate, between 5 and <7.5; and high, ≥7.5[10]; and for hip fracture it was considered high risk ≥3%[11].

Statistic analysis

The risk of fracture was studied by means of a survival analysis: the Kaplan-Meier estimator was calculated, and a proportional hazard model (Cox regression) was made between users and non-users of bisphosphonates, and among patients with previous tamoxifen or without tamoxifen, adjusting for risk covariates. The proportionality of the risk over time was checked. Comparisons between groups were made using the Student's T-test or Chi-Square. The analyzes were performed with SPSS version 23 and with R 3.5.3 using the foreign, plyr, survminer, Hmisc, dplyr, ggplot packages2.

Results

A total of 943 postmenopausal patients on AI treatment were included in the study. Of these, 51 patients (5.4%) suffered an incident fracture (Figure 1). The majority of fractures occurred during treatment with AI although 21.5% occurred during the first year post therapy. 82.4% of fractured patients took letrozole, 15.7% exemestane and 1 patient took anastrozole. The majority of incident fractures detected were vertebral (29.4%) and Colles (31.4%) (Figure 1).

The characteristics of fractured patients are shown in table 1. Most fractured patients (78.5%) were in the overweight range (BMI >25-29.9 kg/m^2) (n=17) or obesity (BMI >30 kg/m^2) (n=24). All humerus fractures oc-

curred in patients with a BMI >28 kg/m^2. Only 2 patients were underweight (BMI <18.5 kg/m^2).

86.3% of the patients were diagnosed with osteopenia or osteoporosis at the time of the fracture, being a key risk factor for the fracture associated with AI. There were no significant differences in fracture risk between patients with and without antiresorptive treatment: HR=1.75 [95% CI: 0.88 to 3.46] (Figure 2). It should be noted that patients with incident fractures treated with bisphosphonates had a significantly lower BMI than patients with fracture and without bisphosphonates [mean (SD): 26.4 (6.2) vs. 30.9 (5.2), respectively; p=0.01]. No differences were found in the other parameters analyzed: age, previous chemotherapy and previous falls.

29.4% (n=15) of the patients had had falls prior to the fracture. Of these, 6 had a vertebral fracture and 8 suffered Colles fracture.

Of all the B-ABLE cohort, 293 previously took tamoxifen and 4.1% suffered a fracture. On the other hand, 650 did not receive prior tamoxifen and 6% fractured (Figure 3). There were no significant differences in the risk of fracture among patients who had previously received tamoxifen treatment compared to those who did not (HR=1.00 [95% CI 0.39 to 2.56]).

VitD levels at baseline had a mean of 17.39±8.2 ng/ml. All patients were treated with VitD at the start of AI treatment, with a mean of 48.69±42.11 ng/ml at 3 months of treatment. Thus, at the time of the incident fracture, all patients had optimal levels of VitD with a mean of 47.7±27.18 ng/ml.

Figure 1. Flowchart of breast cancer patients treated with AI (B-ABLE cohort) with incident fracture

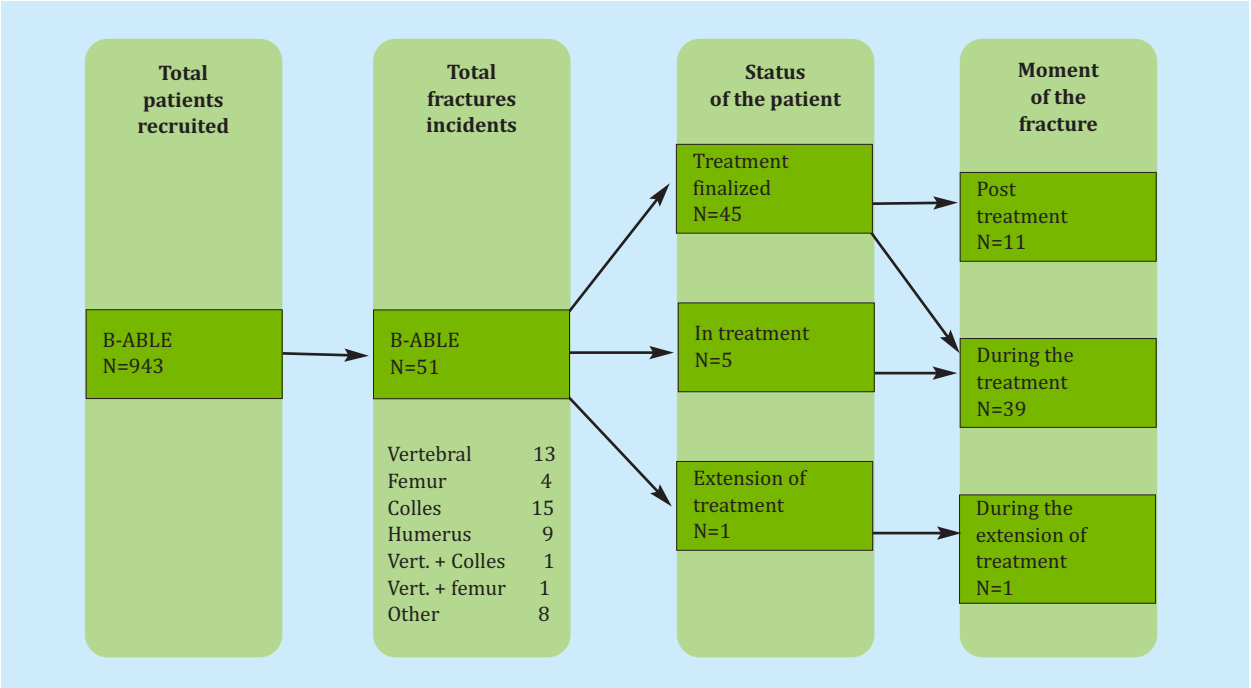
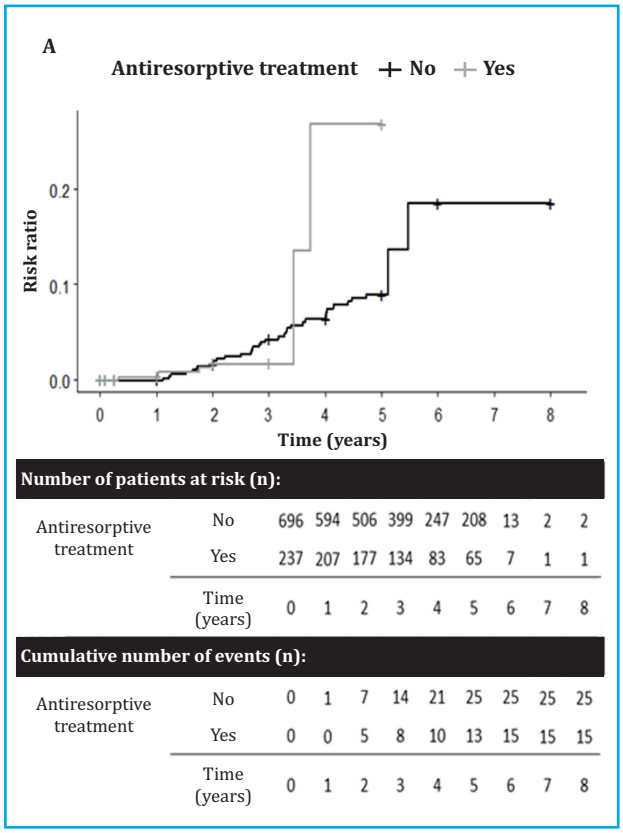


Table 1. Characteristics of the patients at the time of the incident fracture

Characteristics (N=51)	Mean ± SD	n (%)
Mean age (years)	64.45 ± 8.7	
BMI mean (kg/m^2)	29.3 ± 5.8	
Family history of fracture		16 (31.4%)
Previous falls		15 (29.4%)
Mean levels of 25(OH) vitamin D (ng / ml)	47.7 ± 27.18	
Half levels of β-CTX (ng/ml)	0.479 ± 0.25	
Osteoporosis/osteopenia		Osteopenia: 34 (66.7%) Osteoporosis: 10 (19.6%)
Prior tamoxifen		12 (23.5%)
Prior chemotherapy		34 (66.7%)
Antiresorptive treatment		BF: 17 (33.3%)
		Denosumab: 1 (2%)

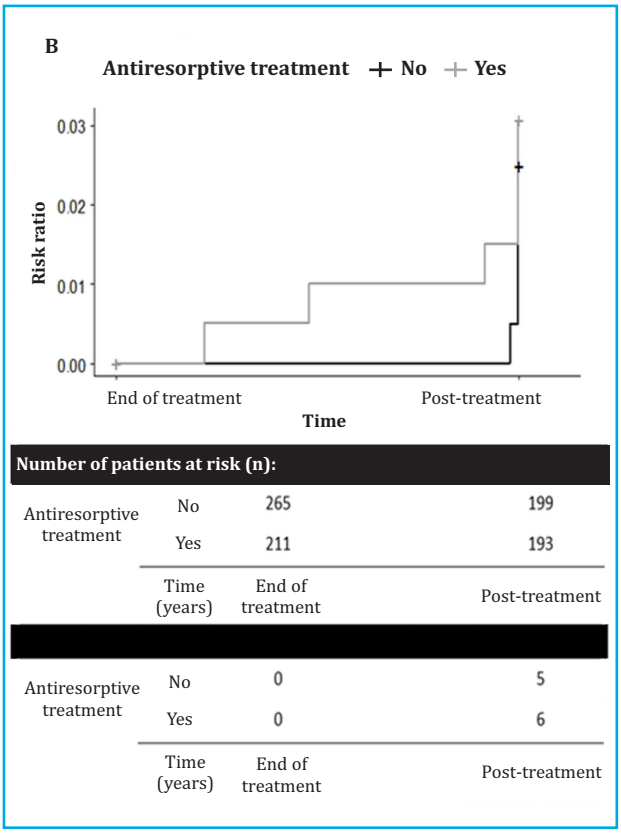
SD: standard deviation; BMI: body mass index; BF: bisphosphonates.

Figure 2. Graph of the cumulative risk of fracture events in study groups (with or without treatment with bone antiresorptives) according to the risk of fracture. The graphs show the Kaplan-Meier curves that set out the study results in terms of cumulative risks. (A) during AI treatment (B) during post treatment



Number of patients at risk (n):										
Antiresorptive treatment	No	696	594	506	399	247	208	13	2	2
	Yes	237	207	177	134	83	65	7	1	1
	Time (years)	0	1	2	3	4	5	6	7	8

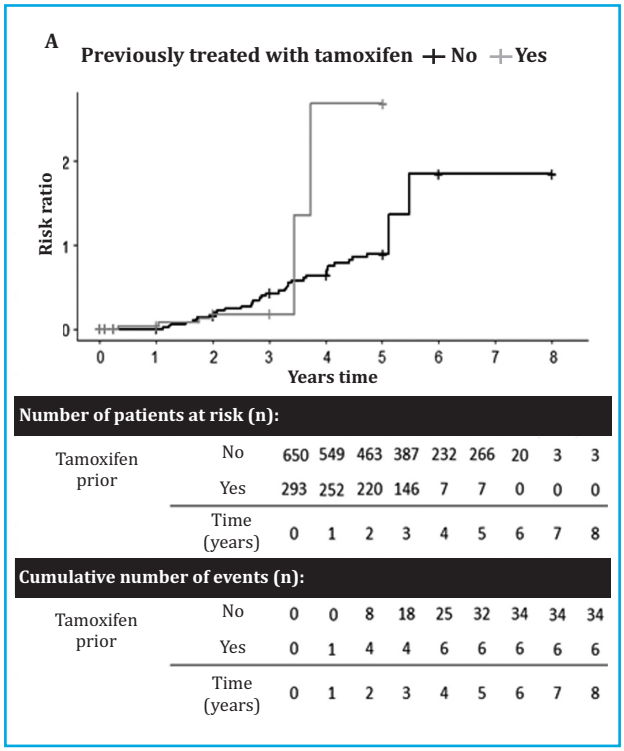
Cumulative number of events (n):										
Antiresorptive treatment	No	0	1	7	14	21	25	25	25	25
	Yes	0	0	5	8	10	13	15	15	15
	Time (years)	0	1	2	3	4	5	6	7	8



Number of patients at risk (n):			
Antiresorptive treatment	No	265	199
	Yes	211	193
	Time (years)	End of treatment	Post-treatment

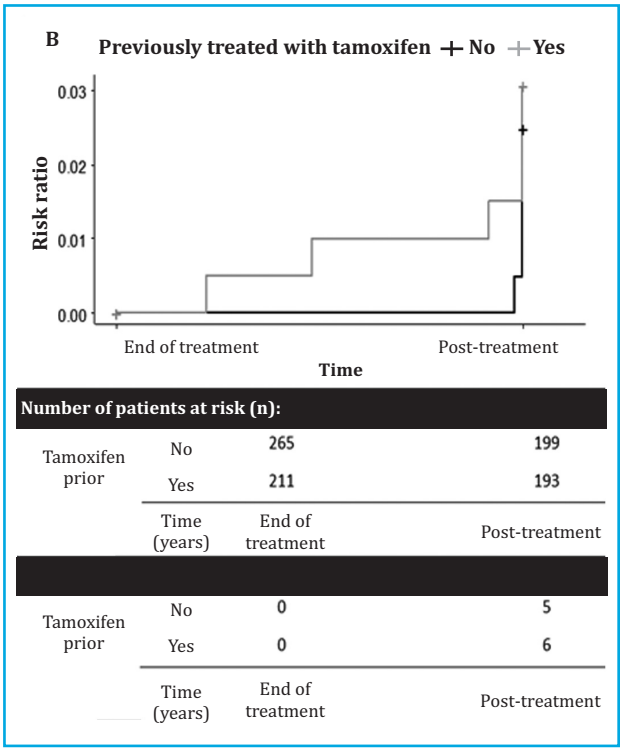
Antiresorptive treatment	No	0	5
	Yes	0	6
	Time (years)	End of treatment	Post-treatment

Figure 3. Graph of the cumulative risk of fracture events in study groups (with or without prior treatment with tamoxifen) according to the risk of fracture. The graphs show the Kaplan-Meier curves that represent the results of the study in terms of cumulative risks. (A) during treatment with aromatase inhibitors, (B) in the post-treatment



Number of patients at risk (n):										
Tamoxifen prior	No	650	549	463	387	232	266	20	3	3
	Yes	293	252	220	146	7	7	0	0	0
	Time (years)	0	1	2	3	4	5	6	7	8

Cumulative number of events (n):										
Tamoxifen prior	No	0	0	8	18	25	32	34	34	34
	Yes	0	1	4	4	6	6	6	6	6
	Time (years)	0	1	2	3	4	5	6	7	8



Number of patients at risk (n):			
Tamoxifen prior	No	265	199
	Yes	211	193
	Time (years)	End of treatment	Post-treatment

Tamoxifen prior	No	0	5
	Yes	0	6
	Time (years)	End of treatment	Post-treatment

According to the normal values of the beta isomer of the carboxyterminal telopeptide of collagen I (β-CTX) in the serum of premenopausal healthy women in the Spanish population (0.064-0.548 ng/ml)[12], 33% of fractured patients had levels of β-CTX above normal. In addition, if the total of 51 patients with fractures exclude those treated with antiresorptives, the mean of β-CTX was at levels above normal (0.585±0.228 ng/ml).

The calculation of the absolute risk of major osteoporotic and hip fractures in the next 10 years, using the FRAX® tool in patients with incident fractures, is shown in table 2. High-risk FRAX values of main fracture were detected (≥7.5) and hip fracture (≥3) in 13 and 8 patients, respectively (Figure 4). In addition, when comparing the means with the B-ABLE patients without incident fracture (Table 3), the average FRAX in the fractured patients was higher than the patients without fracture.

Discussion

AIs produce a deleterious effect on bone tissue that has already been demonstrated in the clinical trials of reference[5]. However, there is little data from prospective non-randomized clinical studies in the usual clinic. This study has focused on the evaluation of the risk factors for incident fracture in the B-ABLE cohort, which includes postmenopausal women with RE (+) breast cancer treated

Table 2. Values of the FRAX® tool for the calculation of fracture risk at 10 years in patients with fracture of the B-ABLE cohort

	Basal FRAX for major fracture	Basal FRAX for major fracture with DXA	FRAX hip	FRAX hip with DXA
Mean ± SD	5.88 ± 4.34	5.9 ± 4.25	1.89 ± 2.75	1.64 ± 2.52
Median	4.4	4.5	0.8	0.6
Minimum	1.4	1.2	0.1	0
Maximum	20	19	15	13

SD: standard deviation; DXA: bone densitometry.

Figure 4. FRAX values of each patient in the study of: A) major fracture and B) hip fracture, taking into account BMD. The horizontal lines of each figure show the threshold established for the risk of fracture at 10 years. Baseline FRAX thresholds for major fracture were: low risk, <5; intermediate, between 5 and <7.5; and high, ≥7.5. The high risk thresholds for hip fracture were ≥3

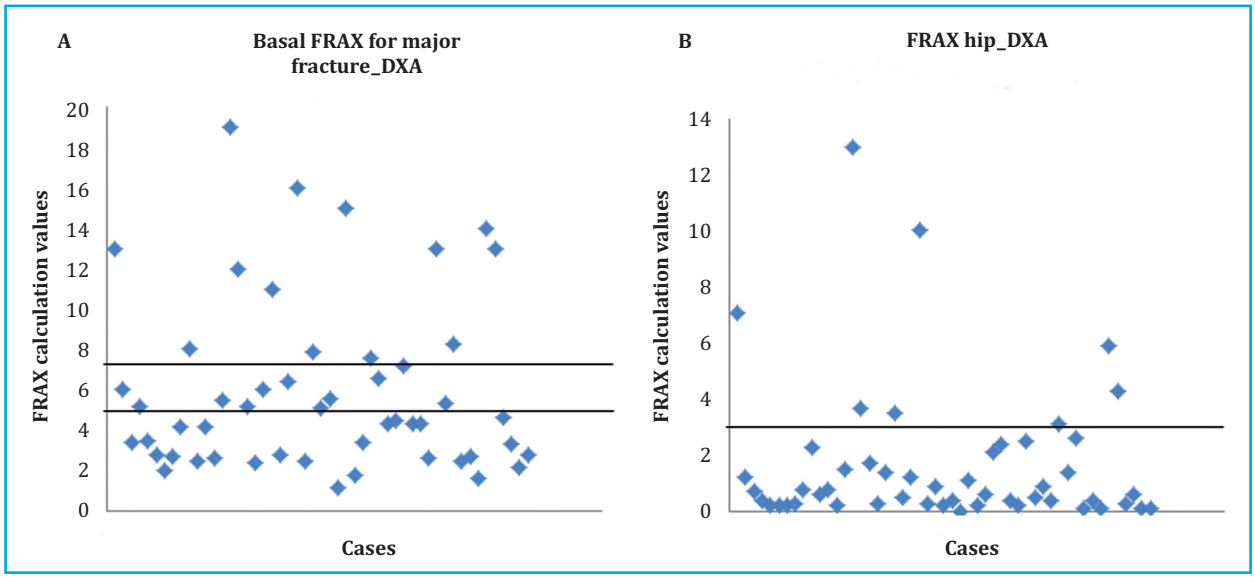


Table 3. Values of the FRAX® tool for the calculation of the fracture risk at 10 years in patients without an incident fracture of the B-ABLE cohort (N=583)

	Basal FRAX for major fracture	Basal FRAX for major fracture with DXA	FRAX hip	FRAX hip with DXA
Mean ± SD	4.92 ± 4.6	4.73 ± 4.15	1.35 ± 2.68	1.04 ± 2.26
Median	3.4	3.3	0.5	0.4
Minimum	0.9	0.9	0	0
Maximum	37	42	29	33

SD: standard deviation; DXA: bone densitometry.

with aromatase inhibitors. The main risk factor detected is the diagnosis of osteopenia or osteoporosis followed by high β-CTX values. Overweight also emerged as a risk factor for the identification of patients with humerus fracture. Likewise, the calculation of FRAX was useful to identify some patients at high risk of main and hip fractures.

All patients in the B-ABLE cohort started treatment with vitamin D supplements from the moment they were included in the study if they had values below 30 ng/ml and, therefore, in most cases vitamin levels D were placed at optimal values during the period of AI therapy. Thus, 86.3% of the patients had vitamin D values greater than 20 ng/ml at the time of the fracture, with an average of 47.7 ng/ml. This rules out sub-optimal levels of vitamin D as a risk factor for fractures in these patients. It should be noted that most of the patients (66.6%) had levels below 20 ng/ml at the time of initiating AI therapy, so we cannot know if these low levels could affect future fractures.

In addition, patients at high risk of fracture at baseline were treated with bone anti-resorptives at the outset of AI therapy, so due to antiresorptive treatment, the risk of fracture decreased. This was thus equated with the incidence of fracture in patients not receiving antiresorptive treatment. These data are in line with a recent study in the SIDIAP cohort (Information System for the Development of Research in Primary Care), in which women treated with bisphosphonates significantly reduced their risk of suffering an osteoporotic fracture[4]. However, more than 30% of the fractures were detected in patients treated with antiresorptives. Interestingly, these women treated with bisphosphonates had a lower BMI than women without antiresorptive treatment.

Although it is generally accepted that having a history of previous falls is a relevant predictor of osteoporotic fracture risk[13], more than 70% of the patients in our cohort did not report falls prior to the incident fracture. It should be noted that in patients with an incident fracture during AI treatment and who reported a history of falls, the most frequent fracture was the vertebral and/or Colles fracture.

Nor have differences in the risk of fracture been detected between patients previously treated with tamoxifen and those who only received AI. However, it was not possible to rule out a possibly insufficient sample size to detect these differences.

The risk of fracture was also assessed with the FRAX tool at baseline (prior treatment with AI), placing most of these patients at intermediate/low risk levels at the time they enter the study. A limitation of the tool is that it does not take into account treatment with aromatase inhibitors, possibly causing the risk of fracture to be underestimated in our cohort. In any case, 25% of patients with fractures had high risk values, so this index could be taken into account when detecting risk patients.

In conclusion, the diagnosis of osteopenia or osteoporosis, along with elevated levels of β-CTX could detect patients treated with AI with a high risk of suffering an incident fracture. Previous treatment with tamoxifen does not seem to affect the risk of fracture.

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Conflict of interests: Authors declare no conflict of interests.

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Differences in bone mineral metabolism normocalcemic primary hyperparathyroidism with respect to classical primary hyperparathyroidism

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Summary

Objective: Normocalcemic primary hyperparathyroidism is a less known variety of classical primary hyperparathyroidism. In this paper, we present its clinical expression and data related to bone mineral metabolism, both analytically and densitometrically, comparing them with a group of patients with classic primary hyperparathyroidism, with hypercalcemia.

Material and methods: Study of cases and controls where we consider case of patients with normocalcemic primary hyperparathyroidism (n=25) and control (n=25) of patients with primary hyperpartyroidism with hypercalcemia (classical primary hyperparathyroidism). A complete clinical assessment was carried out with clinical data collection and 24h blood and urine analytical determinations were performed, as well as estimating bone mineral density and trabecular bone score by densitometry (dual x-ray absorptiometry, DXA) and ultrasound parameters in the calcaneus.

Results: In this clinical study, patients with classic primary hyperparathyroidism only show a higher prevalence of urolithiasis (OR: 9.333; 95% CI: 1.50-82.7) compared to patients suffering from a normocalcemic primary hyperparathyroidism. In all other clinical, analytical, densitometric and ultrasonographic parameters, there are no statistically significant differences between the two groups.

Conclusions: Apart from serum calcium levels and the prevalence of urolithiasis, normocalcemic hyperparathyroidism is indistinguishable from classical hyperparathyroidism.

Key words: hyperparathyroidism, primary, normocalcemic, densitometry, quantity, quality, bone.

Introduction

Primary hyperparathyroidism (HPT) is a very common bone mineral metabolic disease consisting of autonomous overproduction of parathyroid hormone (PTH), which leads to an increase in serum calcium[1]. It is the most frequent cause of hypercalcemia.

A lesser known clinical variant of HPT is the so-called "normocalcemic primary hyperparathyroidism" (NHPT), which has normal blood calcium levels and elevated parathyroid hormone (PTH) values, not knowing the mechanism by which this differential fact occurs[2-4]. These patients do not have clear causes that justify secondary elevations of PTH such as chronic renal damage[5], vitamin D deficiency (less than 30 ng/ml)[6], renal hypercalciuria or drugs[7]. Although NHPT was first formally recognized in the Third International Workshop on the Management of Asymptomatic Primary Hyperparathyroidism in 2008[8], all clinical features are not yet known, particularly with regard to its epidemiology, natural history, management and prognosis[9,10]. Therefore, this clinical variety of the disease is less studied[11] and there is less bibliography. All of which has motivated us to carry out this study.

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Material and methods

This is a case-control study, in which cases patients with primary normocalcemic hyperparathyroidism (PNPH) are considered, and controls those patients with a primary hyperparathyroidism that has attended with hypercalcemia and which we will call classical primary hyperparathyroidism (CHPT). The diagnosis of one or the other clinical picture was made following the criteria established by consensus[12]. All patients were given a questionnaire to collect clinical data, designed for this purpose.

Sample collection and laboratory techniques

Blood and urine samples were collected in the morning, between 8:00 and 9:00, after a fasting night. Blood was collected in the appropriate specific tubes for each determination, with the least possible venous compression, and centrifuged at 1,500 g for 10 minutes. The serum was separated into aliquots and stored within one hour of extraction at -20° C until the biochemical analyzes were carried out, although most of them were done on the same day as the extraction.

Glucose, urea, creatinine, calcium, inorganic phosphorus, total proteins, total cholesterol and its fractions and triglycerides were measured using standardized and automated colorimetric techniques in an auto-analyzer (Kodak Ektachem Clinical Chemistry Slides). The serum calcium was corrected according to total proteins by means of the following formula:

Corrected calcium = previous calcium (mg/dl)/[0.55 + total protein (g/l)/16].

Tartrate resistant acid phosphatase (TRAP) was determined by spectrophotometry. Glomerular filtration (GF) was calculated from the MDRD (Modification of Diet in Renal Disease) formula[13] and the existence of renal insufficiency with GF values below 60 ml/m/m^2 was considered[14].

Serum levels of 25(OH) vitamin D (25HCC) were measured by immunochemiluminescence, according to the Nichols method (Nichols Institute Diagnostics, San Clemente, California, USA). This method has an intra-assay coefficient variation of 3.0-4.5% and intersession of 7.1-10.0%. The values given by the laboratory as normal range between 10 and 68 ng/ml. Serum parathyroid hormone (PTH) concentrations for the intact molecule were determined by immunochemiluminescence, according to the Nichols Advantage method. The normal adult level ranges from 6 to 40 pg/ml, with an inter-assay variation coefficient of 7.0-9.2%. Propeptides of the amino-terminal fraction of collagen type I (P1NP) and blood beta-crosslaps were measured by previously described techniques[15-18]. The remaining biochemical parameters were determined by colorimetric techniques. Urine was collected for 24 hours and calcium, phosphorus and creatinine were measured by automated colorimetric methods.

In patients in the case group (NHPT) with 25HCC values below 30 ng/ml, 25,000 IU of cholecalciferol was prescribed every 15 days and analysis of PTH, calcium and 25HCC was repeated at 3 months, in order to carry out differential diagnosis with hyperparathyroidism secondary to vitamin D deficiency. Once this was ruled out, baseline analysis was considered for the study.

The diagnosis of depression was obtained after a thorough review of the clinical history of all patients, both hospital and primary care.

Ultrasound readings in the calcaneus

Ultrasound parameters were estimated in the calcaneus of the dominant foot, using a Sahara® Hologic® ultrasound (Bedford, Massachusetts, USA). This device measures both the ultrasonic broadband attenuation (BUA), and the speed of sound (SOS) in the region of interest of the calcaneus. The BUA and SOS values are combined into a single parameter called the Quantitative Ultrasound Index (QUI), also known as the consistency index, which is obtained through the formula: QUI = 0.41(SOS) + 0.41 (BUA) – 571. The T-score values were calculated from the values published as normal for the Spanish population[19].

Bone mineral density (BMD)

BMD was measured by dual x-ray absorptiometry (DXA), both in the lumbar spine (L2-L4) and in the proximal limb of the femur, with a Hologic Discovery® densitometer, (Hologic Inc. Waltham, USA). Its accuracy is 0.75-0.16%. The measurements were made by the same operator, so there was no inter-observer variation.

The T-score values were calculated from the values published as normal for the Canary Island population[20].

Trabecular bone score (TBS)

All TBS measurements were carried out using the TBS iNsight Software program, version 2.0.0.1 (Med-Imaps, Pessac, France). The software uses the image previously obtained by DXA in the same region of interest of the lumbar spine L2-L4. The T-score values were calculated from the reference values obtained for the Spanish population[21].

Ethics

The study was carried out following the norms of the Declaration of Helsinki[22] and was approved by the Ethics Committee of the Insular University Hospital. All patients were informed of the objectives of the work and their informed consent was requested.

Statistic analysis

To carry out the statistical study, the R program was used. Initially we analyzed the numerical variables, studying whether or not they followed a normal distribution. Later we carried out a descriptive study. Categorical variables were summarized by percentages, and numerical variables by means and typical deviations. To study the possible associations between categorical variables, the chi-square independence test was used, and as a measure of association the odds ratio (OR) with a 95% confidence interval (95% CI). In those cases where there were cells with less than 5 cases, the exact Fischer test was applied.

To assess the association between a quantitative variable and a categorical variable, Student's t test or ANOVA (if there were more than 2 categories) were used for normal distribution variables, or the non-parametric Mann-Whitney U test for the non-normal to study the degree of association or independence of 2 quantitative variables. We use correlation techniques to assess the strength of the association between the variables.

In all cases the level of significance was considered at 5% ($p<0.05$).

Results

Table 1 shows the baseline characteristics of the patients included in the study. Initially, 30 patients were included

in each group, but they completed the study and finally gave their informed consent 25 patients with HPTN and 25 patients with HPT. This table shows the continuous (numerical) variables. There were no statistically significant differences in any of the variables that we grouped as "baseline characteristics" in table 1, which were: age, height, body mass index (BMI) and size. Therefore, it was not necessary to adjust the remaining parameters studied in our work by any of these variables.

Table 2 presents the clinical characteristics and prevalence of some diseases in both groups of patients studied. Most of the patients were women, with only 4 men being collected in the 25 patients with HPT, which is 15.3%, and 2 men in the group of patients with PNHT, 8% of that group. These differences were not statistically significant (p=0.667). Nor did we obtain statistically significant differences in the prevalence of chronic renal failure, arthralgia, depressive syndrome, or in the prevalence of AHT between the two groups. The only clinical data that showed statistically significant differences between both groups was urolithiasis, which was more frequent in patients affected by the classic form of HPT.

Table 3 shows some biochemical parameters related to bone mineral metabolism. There were no statistically significant differences in renal function (urea, creatinine, uric acid) or in the biochemical markers of bone remodeling, both those of formation and bone resorption (type I procollagen, osteocalcin, tartrate-resistant acid phosphatase and beta-crosslaps), and also at serum levels of PTH and 25(OH) vitamin D.

Table 4 shows the values obtained by means of bone densitometry, both in the lumbar spine (L2-L4) and in the proximal limb of the femur in its different anatomical locations. In all cases the T-score was also calculated, obtained from the normal values of the Spanish population. This same table shows the values of the TBS technique, also calculating the corresponding T-score, based on the normal values of the Spanish population.

Table 5 shows the prevalence of osteoporosis, as well as fragility fractures. There were no statistically significant differences in either the prevalence of densitometric osteoporosis or that of fragility, total or hip fractures, nor in the number of falls between both groups of patients with primary hyperparathyroidism.

Discussion

The NHPT is a rare entity and has consequently received less study. The possible differences with respect to the other classic clinical form of HPTC are not known. In fact, the first recognition of classical HPT as a distinct entity was made at the Third International Workshop on the Management of Asymptomatic Primary Hyperparathyroidism in 2008[8].

Our objective was to try to identify possible differences between the two forms of clinical presentation of HPT, especially in aspects related to bone involvement: prevalence of osteoporosis, involvement of the amount of bone mass measured by bone densitometry (BMD), of bone quality, which we estimated by trabecular bone score (TBS), a relatively recent technique and using software makes an alternative assessment of lumbar spine densitometry, analyzing the quality of trabecular connections[23-26]. This is a complementary method to classical bone densitometry, since it allows the evaluation of

Table 1. Baseline characteristics of both groups studied, patients with normocalcemic HPT (NHPT) and classic primary hyperparathyroidism (CPHPT)

Variable	NHPT	CPHPT	P value
Number	25	25	
Age (years)	67.3 ± 10.2	63.4 ± 11.3	0.205
Height (cm)	160.2 ± 8.4	157.2 ± 9.7	0.244
Weight (kg)	75.7 ± 19.8	74.8 ± 12.5	0.850
BMI (kg/m^2)	30.2 ± 3.6	29.6 ± 8.2	0.758
Wingspan (cm)	158.5 ± 12.3	162.9 ± 8.1	0.143

Table 2. Distribution of sexes and comparison of the prevalence of some clinical data between both groups studied, patients with normocalcemic HPT (NHPT) and classic primary hyperparathyroidism (CPHPT)

Variable	CPHPT N=25	NHPT N=25	OR (IC 95%)	Chi-square	P value
Gender: men, n	4	2	2.190 (0.363-13.219)	0.758	0.667*
Presence of CRF, n	5	1	6.000 (0.647-55.6)	3,030	0.189*
Arthralgias, n	11	14	0.617 (0.202-1.886)	0.720	0.396
Depressive syndrome, n	14	12	1.279 (0.453-4.197)	0.3121	0.571
Urolithiasis, n	7	1	9.333 (1.50-82.7)	5.357	0.049*
AHT, n	19	16	1.781 (0.521-6.085)	0.857	0.355

*: Fischer's exact test was applied as there were cells with less than 5 cases; CRF: chronic renal failure; AHT: arterial hypertension.

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Table 3. Biochemical data obtained in both groups studied, patients with normocalcemic HPT (NHPT) and classic primary hyperparathyroidism (CHPT)

Variable	NHPT	CPHPT	P value
Urea (mg/dl)	40.2 ± 18	40.2 ± 16.9	0.989
Creatinine (mg/dl)	0.9 ± 0.3	1 ± 0.3	0.483
Calcium (mg/dl)	9.9 ± 0.4	11 ± 0.5	0.001
Phosphorus (mg/dl)	3.1 ± 0.4	2.7 ± 0.4	0.007
Total proteins (g/l)	7.1 ± 0.3	7.1 ± 0.4	0.728
Calcium corrected (mg/dl)	10 ± 0.5	11.1 ± 0.5	0.001
Uric acid (mg/dl)	5.1 ± 1.5	5.3 ± 1.5	0.662
Calciuria (mg/24h)	168.2 ± 114.2	235.3 ± 153.8	0.15
Phosphaturia /mg/24h)	635.7 ± 305.4	747.1 ± 279.1	0.13
Biochemical markers of bone remodeling and hormones			
P1NP* (mg/ml)	59.1 ± 33.8	77.2 ± 52.6	0.185
Osteocalcina (ng/ml)	33.5 ± 17.5	35.3 ± 15.6	0.711
Beta-crosslaps (ng/ml)	0.6 ± 0.3	0.8 ± 0.6	0.144
TRAP[§] (UI/l)	3.1 ± 0.9	3.1 ± 0.8	0.945
PTH[¥] (pg/ml)	119 ± 33	122 ± 20.7	0.701
Vitamin D (25HCC)[#] (ng/ml)	23.5 ± 9.7	21.9 ± 9	0.539

*: aminoterminal type I procollagen; [§]: tartrate-resistant acid phosphatase; [¥]: intact parathyroid hormone; [#]: 25 hydroxycholecalciferol.

Table 4. Densitometric values in lumbar spine and proximal limb of the femur, TBS and ultrasound in the calcaneus in both groups studied, patients with normocalcemic HPT (NPHPT) and classic primary hyperparathyroidism (PHPT). Ultrasound in the calcaneus

Variable	NHPT	CPHPT	P value
L2L4 (g/cm^2)	0.922 ± 0.200	0.929 ± 0.168	0.897
T-score L2L4	-1.1 ± 1.5	-1.0 ± 1.3	
Femoral neck (g/cm^2)	0.711 ± 0.114	0.728 ± 0.154	0.661
T-score femoral neck	-1.1 ± 0.9	-1 ± 1.2	
Total hip (g/cm^2)	0.843 ± 0.144	0.860 ± 0.156	0.693
T-score total hip	0.0 ± 1.0	-0.1 ± 1.1	
Trochanter (g/cm^2)	0.630 ± 0.120	0.644 ± 0.120	0.701
T-score trochanter	-0.1 ± 0.9	0.0 ± 0.9	
Intertrochanter (g/cm^2)	0.980 ± 0.171	1.010 ± 0.185	0.643
T-score intertrochanter	0.0 ± 1.0	0.0 ± 1.1	
TBS lumbar spine (g/cm^2)	1.288 ± 0.087	1.276 ± 0.105	0.747
T-score TBS	-1.9 ± 1	-2.1 ± 1.3	
Ultrasound in the calcaneus			
BUA (dB/MgHz)	66.9 ± 16.2	58.4 ± 14	0.148
SOS (m/s)	1,530.8 ± 33.4	1,518.3 ± 21.9	0.263
QUI	84 ± 19.7	75.4 ± 13.4	0.196

TBS: trabecular bone score. Bone trabecular score; BUA: broadband ultrasound attenuation. Ultrasonic Broadband Attenuation; SOS: speed of sound. Speed of sound; QUI: quantitative ultrasound Index. Quantitative Ultrasonic Index.

Table 5. Prevalence of osteoporosis, falls and fragility fractures in both groups studied, patients with normocalcemic HPT (HPTN) and classic primary hyperparathyroidism (CHPT)

	NHPT	CPHPT	OR (IC 95%)	Valor p
Densitometric osteoporosis, n (%)	5 (20%)	5 (20%)	1.000 (0.250 - 3.998)	1.000
Fragility fractures, n (%)	8 (32%)	6 (24%)	1.490 (0.429 - 5.172)	0.529
Falls in the last year, n (%)	6 (25%)	7 (28%)	0.857 (0.240 - 3.056)	0.812
Hip fracture, n (%)	0 (0%)	0 (0%)	Not applicable	Not applicable

aspects more related to bone architecture, being an indirect method of estimating bone quality[23,24,27]. Finally, we used ultrasound, a controversial method, which some authors recommend to measure bone quality[28,29].

We have not found statistically significant differences in the variables analyzed between both groups of patients with HPT, with the only exception of serum calcium values, the variable that distinguishes between one group and another. It is well known that HPT in its traditional form occurs more frequently in women and this same finding has been found in our study. Nor were differences observed in the prevalence of falls, chronic renal failure, the clinical presentation of arthralgia, depressive syndrome or high blood pressure (AHT). In contrast, patients who had CHPT presented a higher prevalence of kidney stones. Few studies analyze these clinical data in the literature. We found a series of cases published by Cusano et al. We included 9 patients who showed clinical and biochemical data very similar to those obtained in our work[3], while in another series we obtained conclusions precisely opposite to ours. In the series reported by Amaral et al. with 33 cases, an 18% prevalence of kidney stones was found, the same prevalence as the control group formed by patients with CHPT[30].

All these clinical manifestations (arthralgias, depression) or the association of other conditions such as high blood pressure or chronic renal failure can be observed in the HPT[1,31-36], although today, with the development of laboratory techniques and programs health prevention that include analytical determinations, HPT is usually diagnosed as an asymptomatic hypercalcemia, without any other symptoms[1,35,36]. Since precisely hypercalcemia is the guiding sign in the diagnosis of HPT, in the case of NHPT the diagnosis is more complicated and is reached by exclusion, after a more detailed study[2-4].

The results obtained on bone mineral metabolism indicate that bone remodeling does not differ in the two forms of HPT. Similar results to ours have been described in other studies[2-4,11,30].

We did detect statistically significant differences in PTH or vitamin D either. It should be noted that the average values of vitamin D, measured by its reserve metabolite, 25HCC[37], were low, in the range of vitamin D insufficiency, which is defined as serum values of 25HCC below 30 ng/ml[38,39]. This finding has been corroborated in other studies that coincide with our results[4,36,40,41].

Nor have we observed a different behavior of the bone in both groups of patients, since BMD values both in the lumbar spine (L2-L4) and in the proximal limb of the femur in all locations (femoral neck, total hip, trochanter and intertrochanter) were similar in both groups, thus affirming that in the normocalcemic primary HPT there are no differences in bone mineral density with respect to the HPTC. We have obtained the same finding when studying the TBS, which has been studied in patients with HPT and has shown lower values than the controls[24], and may indicate involvement of the trabecular structure and therefore of bone quality[23,25,26,41]. Regarding the existence of osteoporosis due to densitometry or the appearance of fragility fractures, we did not obtain statistically significant differences between both groups of patients with HPT. In fact, the existence of densitometric osteoporosis was observed the same number of patients in each group. No hip fracture event was observed. It does not appear, therefore, that there are clinical differences in bone involvement in patients with NHPT with respect to CHPT.

Our study's main limitation is the small sample size, due to the difficulty of detecting cases. It is noteworthy that NHPT is a condition whose incidence and actual prevalence are unknown. However, when reviewing the literature, we have verified that it is a very rare entity. The number of cases in the different reported series is also low[6,10-12,30,35].

Conflict of interests: Authors declare no conflict of interests.

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Effects of bazedoxifene treatment on the bone quality of ovariectomized rats

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Summary

Objetive: Bazedoxifene is a 3rd generation SERM with agonistic effects on the bones, uterus and breast tissue. Our goal has been to study the effects of bazedoxifene on bone quality of an experimental group of ovariectomized rats.
Material and methods: 3 groups of 15 6-month-old Wistar female rats were used: a control group, a group of untreated ovariectomized rats and a group of ovariectomized rats treated with bazedoxifene (0.33 mg/kg/day). After 8 months we studied the lumbar and femur bone densitometry, the microtomographic parameters, the biochemical markers for bone remodelling and the bone biomechanical parameters.
Results: The ovariectomy depleted the femur and lumbar bone density. After receiving bazedoxifene, the lumbar bone density showed partial healing. Bone remodelling increased recovering bazedoxifene formation levels. Bazedoxifene promoted the recovery of the bone volume fraction (BV/TV), the bone surface density (BS/BV), the trabecular number (Tb.N), the trabecular spacing (Tb.Sp), the trabecular pattern factor (Tb.Pf) and the structural model index (SMI). The cortical surface increased after the ovariectomy and returned to normal levels with the administration of bazedoxifene. The maximum deformation showed before the ovariectomy was also restored, partially cushioning the ovariectomized rats' weight gain.
Conclusions: Our study has shown bazedoxifene positive results on bone quality. This specific drug could be particularly suitable for young postmenopausal women suffering or at risk of suffering osteoporosis.

Key words: bazedoxifene, bone mineral density, bone remodelling, microtomography, biomechanics, endometrial safety.

Introduction

Selective estrogen-receptor modulators (SERMs) are synthetic, nonsteroidal agents with estrogenic agonist-antagonist activity in different target tissues[1]. Their estrogenic responses are mediated by estrogen receptors (α and β). SERMs may present agonistic or antagonistic behavior depending on the tissue type[2,3]. In general, SERMs exhibit agonist activity in the liver, the digestive tube, the skeleton and the heart, but antagonist activity in the breast. In the uterus some SERMs manifest agonist activity while others show an antagonist behavior[1]. Several co-regulatory proteins modify the behavior of the SERMs on gene expression and contribute to their tissue-selective pharmacology.

Tamoxifen is a SERM used as a mammary antiestrogen for preventing and treating breast cancer with estrogen agonistic activity in the uterus. Raloxifene has been used for the prevention and treatment of osteoporosis and prevents breast cancer but presents some estrogenic activity[4]. Bazedoxifene is a 3rd generation SERM with agonistic effects on the bone and additional positive effects on lipids, the uterus and the breast tissue[5,6].

Due to its estrogen agonistic activity on the bone, raloxifene and bazedoxifene are used to treat osteoporosis. Bazedoxifene has the advantage of a greater endometrial safety, therefore it is as well widely used in combination with conjugated equine estrogens for the treatment of endometriosis[7-9].

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Our study focuses on the effects produced by bazedoxifene on the bone. However, we find it interesting to point out that bazedoxifene has also been identified as an effective therapeutical agent against human colorectal cancer[10], breast cancer[11], gastrointestinal cancer[12] and gastric adenocarcinoma[13].

Regarding the effects on the bone, Keating et al.[14] found that bazedoxifene reduced the rates of new vertebr al fractures in patients affected by osteoporosis, as well as the rates of non-vertebral fractures in high-risk patients. Moreover, it is a very well-tolerated drug, without adverse effects on the endometrium or breast tissue[7].

The purpose of our research was to study the effects of bazedoxifene on the bone quality in detail, using an experimental group of ovariectomized rats and long-term treatment (8 months). We examined the lumbar and femoral bone densitometry, the microtomographic trabecular and cortical parameters, the biochemical markers reflecting bone formation and bone resorption and the bone biomechanical parameters.

Material and methods

45 6-month-old Wistar female rats from the Jimenez Diaz Foundation animal facility were used. These rats were kept at a constant temperature of 22ºC, observing 12-hour light-dark cycles and with free access to food and drink. The food was a complete diet for rats and mice (Panlab®, Barcelona, España). The average weight of the rats at the beginning of the study was 333.6±32 g (mean ± standard deviation).

The rats were randomly divided in 3 groups:

1. SHAM group (n=15): the ovariectomy was simulated; 2. OVX group (n=15): ovariectomized rats; OVX + BZD group (n=15): ovariectomized rats, administered 0.33 mg/kg/day of bazedoxifene using a feeding tube for 8 months. The treatment started the day after the ovariectomy had been performed and continued during the following 8 months. Every single treatment was administered according to the EU directives on the protection of animals used for scientific purposes and were approved by the ethics committee of the Institute for Health Research of the Jimenez Diaz Foundation.

The bazedoxifene drug was Conbriza® (Pfizer), donated by Pfizer Laboratories. The dosage was calculated based on the recommended treatment for osteoporosis in humans, 20 mg/day taken orally, therefore the dose of bazedoxifene we used on our rats was 0.33 mg/kg/day through a feeding tube and 0.3 ml of water for each animal.

For surgery, the rats were anaesthetized via intramuscular injections of 0.7 ml of a 1:2 mixture of 2 g/ml of xylazine hydrochloride (Rompun®) and 50 mg/ml of ketamine (Ketolar®). Once anesthetized, all four limbs were immobilized and the area to be operated on was clipped. The animals were in the supine position, leaning on their backs. The bilateral ovariectomy surgery was carried out through an abdominal incision. To remove the ovaries, the uterine horns were identified, one end attached to the ovary and the other to the uterus. When ties were established on either side of the ovary, we proceeded to section and remove them. Once this process finished, the incision was stitched. After 8 months of treatment, the rats were weighed and sacrificed via exsanguination by a heart perfusion under anaesthesia with Isoflurane (Forane®). Through this perfusion, we obtained the blood samples that would be centrifuged at 3,000 r.p.m. for 15 minutes to obtain the serum. This serum was divided into aliquots and frozen at -80ºC, up to the moment the bone remodelling parameters were to be determined.

After extracting their blood, the rats were frozen at -20ºC until bone mineral density measurement had to be taken. The day before such procedure, the rats were introduced in a fridge at -20ºC in order to thaw. Then their right and left femur were amputated using scalpel and tweezer. Once the femurs were extracted and cleaned, we performed a bone mineral densitometry on the left femur and spine at L2, L3 and L4 levels.

Bone densitometry

We proceeded to the determination of the bone mineral densitometry (BMD) of the left femur and the spine at L2, L3 and L4 levels, undergoing a dual-energy X-Ray densitometry (DXA). We used a machine called Piximus (Hologic®, QDR-1000 TM), a specific densitometer for animal and small samples.

The BMD scanning was carried out on the femur entirely and on the whole three vertebrae (L2, L3 and L4), and the results were expressed as the average of the obtained values. The inter- and intra-assay coefficients of variability were <0.53% and <1.2% respectively.

After taking this measurement, the femurs were wrapped in gauze soaked in physiological saline solution and kept frozen at -20ºC until the computerized microtomography was carried out. The right femurs were kept in the same way for biomechanical testing. In these circumstances, the mechanical properties of the bone were not found to significantly change for at least 7 or 8 months. Likewise, no variations have been observed after samples go through up to 5 short freezing-thawing periods[15].

Biochemical markers of bone remodeling

Blood samples were thawed to determine biochemical markers of bone remodeling.

Biochemical markers of bone formation:

- Osteocalcin (BGP): a specific commercial colorimetric immunoassay (ELISA) was used for the determination of osteocalcin levels in rats (Rat-MID™ Osteocalcin, IDS, UK). The sensitivity of the assay was 50 ng/ml, and the inter- and intra-assay coefficients of variability were <5.0% and <6.6%, respectively.

- Procollagen I amino-terminal propeptide (PINP): a specific commercial enzyme immunoassay (ELISA) was used to determine concentrations of PINP in rats (Rat/Mouse PINP, IDS, UK). The sensitivity of the method was 0.7 ng/ml, and the inter- and intra-assay coefficients of variability <5% and <8.2%, respectively.

Biochemical marker of bone resorption:

- Type I collagen carboxy-terminal telopeptide (CTX): a rat-specific ELISA (RatLaps CTX-I ELISA, IDS, UK) was used. The sensitivity of the assay was 2.0 ng/ml and the inter- and intra-assay coefficients of variability of this method were <5.6% and <10.5% respectively.

Microtomography

The left femurs of the rats were sent to the University of Oviedo to study the bone microarchitecture from the computerized microtomography (micro-CT) images got from the bone samples. This analysis was performed in the distal metaphysis of the femur and in a cortical bone ring of its diaphysis.

All samples were scanned on a SkyScan 1174 desktop X-Ray microtomograph (Bruker, Kontich, Belgium). The samples were placed with the long axis perpendicular both to the base of the sample holder and to the X-Ray source. The images were obtained under the following conditions: voltage of the X-Ray source: 50 KV; X-Ray source intensity: 800 µA; use of 1mm aluminum filter; resolution: 17.1 µm; sample rotation step: 0.4°; total rotation: 180°; frame averaging: 2; exposure time: 11,000 ms; approximate scanning time per sample: 3 hours and 50 minutes. 930 tomograms in TIFF format were obtained from each sample.

The flat-field correction was carried out at the beginning of every scan. The tomograms obtained from scanning the samples were reconstructed using the Feldkamp algorithm, modified in the NRecon application, version 1.6.9.16 (Bruker microCT, Kontich, Belgium). The optimal parameters selected were: ring artefact reduction: 8; beam hardening correction: 30´; smoothing: 1.

The scanning and reconstruction parameters used were the same for all samples. After the reconstruction, two different volumes of interest (VOI) were selected using the CTAn application, version 1.14.4.1, (Bruker, Kontich, Belgium) in which to determine the microstructural properties and bone mineral density. In the case of the trabecular bone, a VOI was selected starting at 1 mm from the growth cartilage of the distal metaphysis of the femur (taken as reference section) and occupying 3.4 mm in the proximal direction (a total of 200 images), excluding the cortical bone to be analyzed. For cortical bone analysis, the growth cartilage of the distal metaphysis is again taken as a reference, starting the VOI at 14 mm from it and covering 2.5 mm (150 images). The structural analysis of the VOI is carried out with the software provided with the equipment (CTAn version 1.14.4.1). Once the results of the microstructural parameters were obtained, the CTVol 2.2.3.0 program (Bruker, Kontich, Belgium) was used to visualize the three-dimensional models created with CTAn using the Marching cubes 33 algorithm.

For the trabecular bone, standard cancellous bone morphometric parameters were determined by a 3D analysis of the trabeculae.

The parameters studied for the trabecular bone are detailed below.

Surface and volume relationships:

The bone volume fraction (BV/TV) perfectly reflects the bone loss or gain in the different groups. It is obtained from the basic morphometric indexes, bone volume (BV) and total volume of interest (TV). It is commonly expressed as a percentage. The total area of the trabecular bone (BS) is measured by triangulating the surface of the object. Its relationship with the volume of interest analyzed is known as bone surface density (BS/TV). It is expressed in mm^{-1}, as it is the quotient between an area unit and a volume unit. The bone specific surface (BS/BV) expresses the relationship between the total area of the trabecular bone with the volume occupied only by mineralized bone. Like the previous variable, it is also expressed in mm^{-1}.

Direct metric indices:

The trabecular thickness (Tb.Th) is calculated following a method that occupies with spheres the structure analyzed by distance transformation. It is usually expressed in mm or µm. The trabecular separation (Tb.Sp) is calculated in the same way, but this time occupying the medullary cavities. It is expressed in mm or µm. The trabecular number (Tb.N) means the number of times trabeculae are traversed by an arbitrary path through the volume of interest per unit length. The method is to launch a line through the region of interest and count how many times it crosses trabeculae. It is expressed in mm^{-1}.

Direct non-metric indices:

The trabecular pattern factor (Tb.Pf) quantitatively describes trabecular connectivity. It is an inverse connectivity index (the higher the Tb.Pf value, the less connected the trabeculae are) based on the calculation of a relative convexity or concavity index of the total bone surface, in which the concavity of the trabecular surfaces implies connectivity, while convexity indicates disconnected and isolated structures. The higher the Tb.Pf value, the worse connectivity the trabecular network shows, which implies a decrease in mechanical resistance. It is expressed in mm^{-1} [16]. The structural model index (SMI) shows the relative prevalence of plate-like or rod-like trabeculae, indicating more presence of plates the closer its value get to zero[17]. It is defined in a range of values from 0 to 3, where 0 is an ideal plate-shaped structure and 3 is a cylinder. The degree of anisotropy (DA) is a measure of the symmetry of the object or the presence/absence of structures aligned in a certain direction. It is a dimensionless variable. Zero is total isotropy and 1 is total anisotropy. The different variables were directly measured using methods described in the bibliography[18,19].

Two different analyses were carried out in the cortical region. The first one (endosteum-periosteum separation) allowed us to calculate total volume, bone volume and medullary volume. In the second one we report the porosity of the cortical bone.

Endosteum-periosteum separation: the total volume of the cross section inside the periosteum (VIP) is the mean value of the volume occupied by bone and bone marrow in the analyzed cross sections. It is expressed in mm^3. A low VIP value indicates that there is less bone formation and more resorption, and the other way round if we find a high value. Cortical bone volume (Ct.BV) is the mean value of the volume occupied by bone in the analyzed cross sections. It is expressed in mm^3. The medullary volume (Md.V) is the mean value of the volume occupied by the bone marrow in the analyzed cross sections. It is expressed in mm^3. This value indicates the opposite of VIP.

Porosity parameters studied: cortical bone volume excluding pores (Ct.BV); the ratio between the cortical surface and the volume of the cortical bone without pores (Ct.BS/BV); and the porosity of the cortical bone (Ct.B.Po).

Biomechanics

The right femurs of the rats remained frozen at -80ºC and were thawed prior to the mechanical test for proper preparation. The test was carried out on a universal testing machine. A 3-point bending test was set up, with a spacing of 17.6 mm and an indenter diameter of 5.6 mm. The force was applied perpendicularly to the axis of the bone, in the region of the diaphysis, with an application speed of 10 mm/min (0.17 mm/s). We obtained a load-displacement curve for each sample and we proceeded to calculate the diameter of the diaphysis from the average of 6 different measurements, to minimize the effect of variability.

Analyzed biomechanical parameters:

From the curve resulting from each experiment, different parameters indicating the mechanical characte-

ristics of the samples have been determined[20]: maximum bending force at the time of mechanical failure; displacement at the time of mechanical failure; extrinsic stiffness; breaking energy; maximum tension; maximum deformation; and Young's modulus.

Statistic analysis

The results have been expressed as mean ± standard deviation (SD) of the different parameters. The treatment groups have been compared using the Mann-Whitney test for unpaired samples (Medcal, Belgium). Differences have been considered significant from a value of $p<0.05$.

Results

Figure 1 shows the results obtained in the femur bone mineral density (FBMD) and lumbar bone mineral density (LBMD) of the rats studied. Ovariectomy produced a significant decrease in bone density in the femur and spine. Bazedoxifene treatment partially recovered lumbar density, but not femur density.

Figure 2 shows the levels of the biochemical markers of bone remodeling in the groups of rats studied. As expected, markers of bone formation and resorption (BGP, PINP, and CTX) experienced a significant increase after ovariectomy. Bazedoxifene treatment recovered the basal levels of BGP and PINP, without significant variations in CTX levels.

Figure 3 shows a series of studied quantitative microstructural parameter. Bone volume fraction (BV/TV) and bone surface density (BS/TV) decreased after the ovariectomy, partially recovering after the treatment with bazedoxifene. Bazedoxifene also partially recovered the increase in the trabecular separation (Tb.Sp) produced by the ovariectomy, as well as the decrease in the trabecular number (Tb.N), without acting on trabecular thickness (Tb.Th).

Figure 4 shows the non-metric variables Tb.Pf and SMI and the quantitative variables Conn.Dn and DA in the groups of the studied rats. The trabecular pattern factor Tb.Pf increased significantly in the ovariectomized rats, indicating a significant loss of trabecular connectivity after the ovariectomy. Bazedoxifene treatment partially corrected this loss. The ovariectomy also significantly increased the structural model index SMI, indicating a prevalence of rod-shaped trabeculae, compared to control rats, with a prevalence of plate-shaped trabeculae. Bazedoxifene treatment also partially corrected this variation. The degree of anisotropy decreased significantly after the ovariectomy, increasing after treatment with bazedoxifene to values higher than that of the control rats.

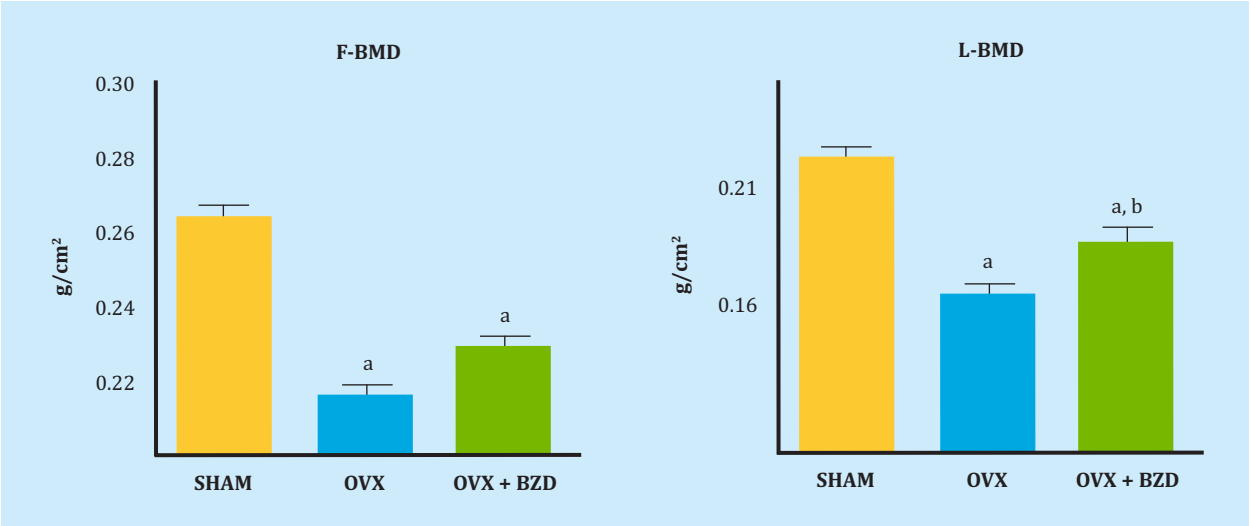
Figure 5 shows the results of bone volume + bone marrow (VIP), cortical bone (Ct.BV) and medullary volume (Md.V) in the cortical. Bone + marrow volume did not seem to have varied significantly after the ovariectomy, but it was lower in the ovariectomized rats treated with bazedoxifene, suggesting an unresolved ovarian failure influenced by this drug. Cortical bone volume (Ct.BV) decreased significantly after the ovariectomy, with bazedoxifene not exerting a positive action. Medullary volume (Md.V) increased after the ovariectomy, remaining constant after bazedoxifene treatment.

Cortical bone volume decreased after the ovariectomy ($p<0.05$), with bazedoxifene treatment not producing any effects.. The relative cortical surface increased after the ovariectomy ($p<0.05$), normalizing after treatment with bazedoxifene. The porosity (Ct.B.Po) decreased significantly after the ovariectomy ($p<0.001$), with bazedoxifene treatment not producing variations.

Maximum displacement, stiffness, break work, maximum tension, and Young's modulus did not vary with the ovariectomy or the break work. The maximum bending force at the time of mechanical failure decreased with the ovariectomy ($p<0.05$), as expected, with no effect from bazedoxifene. The maximum deformation before rupture decreased with the ovariectomy ($p<0.05$), recovering with bazedoxifene treatment.

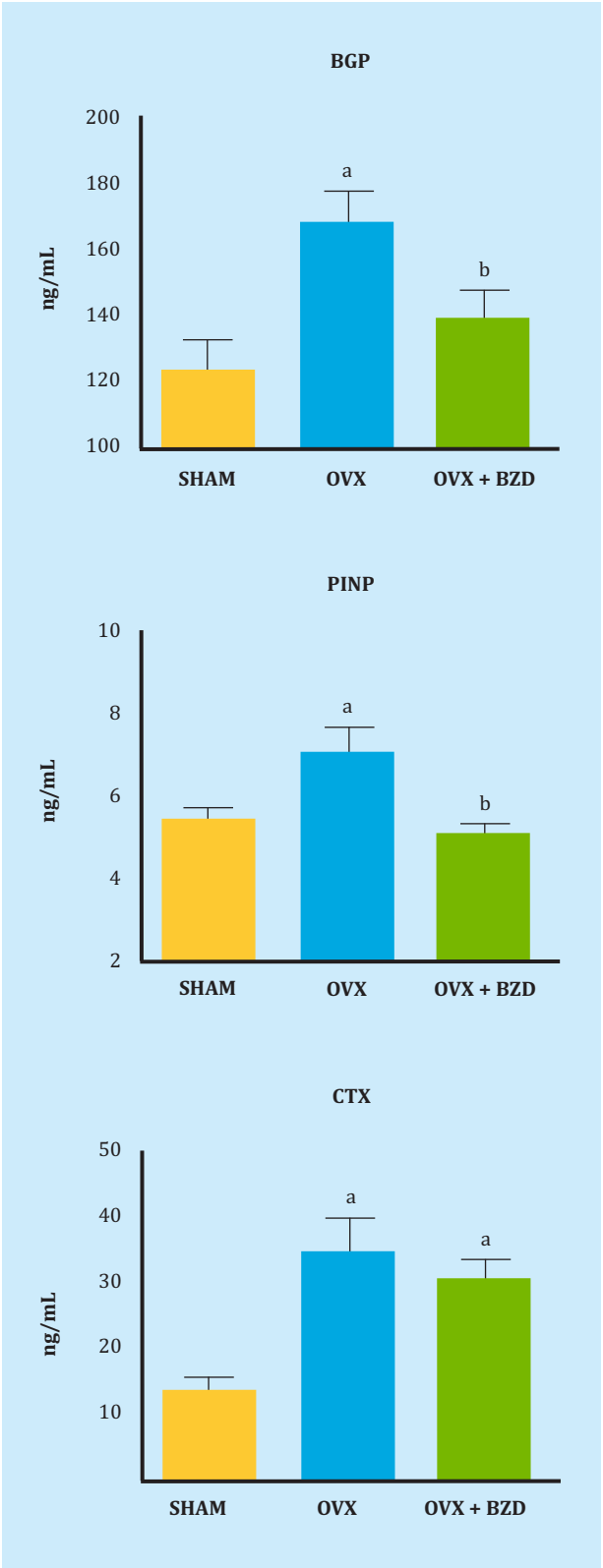
Regarding the weights of the rats, at the end of the experiment the SHAM group weighed 380±25 g, the OVX group 475±30 g (OVX vs SHAM, $p<0.01$) and the group treated with bazedoxifene 425±15 g (BZD vs SHAM, $p<0.05$; BZD vs OVX, $p<0.05$). The ovariectomy made the rats gain weight and the treatment with bazedoxifene partially cushioned this gain.

Figure 1. Femoral bone mineral density (F-BMD) and lumbar bone mineral density (L-BMD) in the 3 groups of rats: SHAM (control), ovariectomized (OVX) and ovariectomized remodel treated with bazedoxifene (OVX + BZD)



F-BMD. a: OVX vs SHAM, $p<0.01$; OVX+BZD vs SHAM, $p<0.01$; **L-BMD. a**: OVX vs SHAM, $p<0.01$; OVX+BZD vs SHAM, $p<0.05$, **b**: OVX+BZD vs OVX, $p<0.05$.

Figure 2. Biochemical markers of bone remodelling: osteocalcin (BGP), aminterminal procollagen I propeptide (PINP) and carboxyterminal collagen I telopeptide (CTX) in the 3 groups of rats: SHAM (control), ovariectomized (OVX) and ovariectomized treated with bazedoxifene (OVX + BZD)



BGP. a: OVX vs SHAM, p<0.01, **b**: OVX+BZD vs OVX, p<0.01; **PINP. a**: OVX vs SHAM, p<0.01, **b**: OVX+BZD vs OVX: p<0.01; **CTX. a**: OVX vs SHAM p<0.01; OVX+BZD vs SHAM p<0.01.

Discussion

According to our results, bazedoxifene treatment partially recovered lumbar bone density, but not femur bone density.

Coinciding with this, Barrionuevo et al.[21] conducted a study including 107 clinical trials in which it could be concluded that there was a significant reduction in vertebral fractures with bazedoxifene. Similarly, Jin et al.[22], studying 41 articles from clinical trials from 2015 to 2019, concluded that bazedoxifene prevents vertebral fractures. Peng et al.[23] conducting a systematic review of studies carried out over 3 and 7 years, and Palacios et al.[24], in a study carried out over 7 years, observed that the incidence of new vertebral fractures was lower in women treated with bazedoxifene than in the placebo group.

Regarding the biochemical markers of bone remodeling, our results show a decrease in the same in BGP and PINP levels after treatment with bazedoxifene, although without changes in PINP. Coinciding with our results, Bueno et al.[25] observed in a study carried out in 7,492 patients that bazedoxifene reduced bone remodeling in postmenopausal Latino women affected by osteoporosis. In this regard, it is important to note that not only the decrease in bone mineral density, but also the increase in bone remodeling is associated with an increased risk of fracture[26], and that changes in osteocalcin levels after 6 months of treatment predicted the changes in bone mineral density observed after 2 years[27].

Regarding bone quality, according to the parameters of the microtomography, our results showed positive effects from the treatment with bazedoxifene on the trabecular parameters BV/TV, BS/TV, Tb.Th, Tb.Sp.Tb.N. Tb.Pf, SMI, DA and Md.V and on the cortical Ct.BS/BV, although the basal values of the rats from the control group were not recovered in all cases, but they did improve compared to the ovariectomized ones.

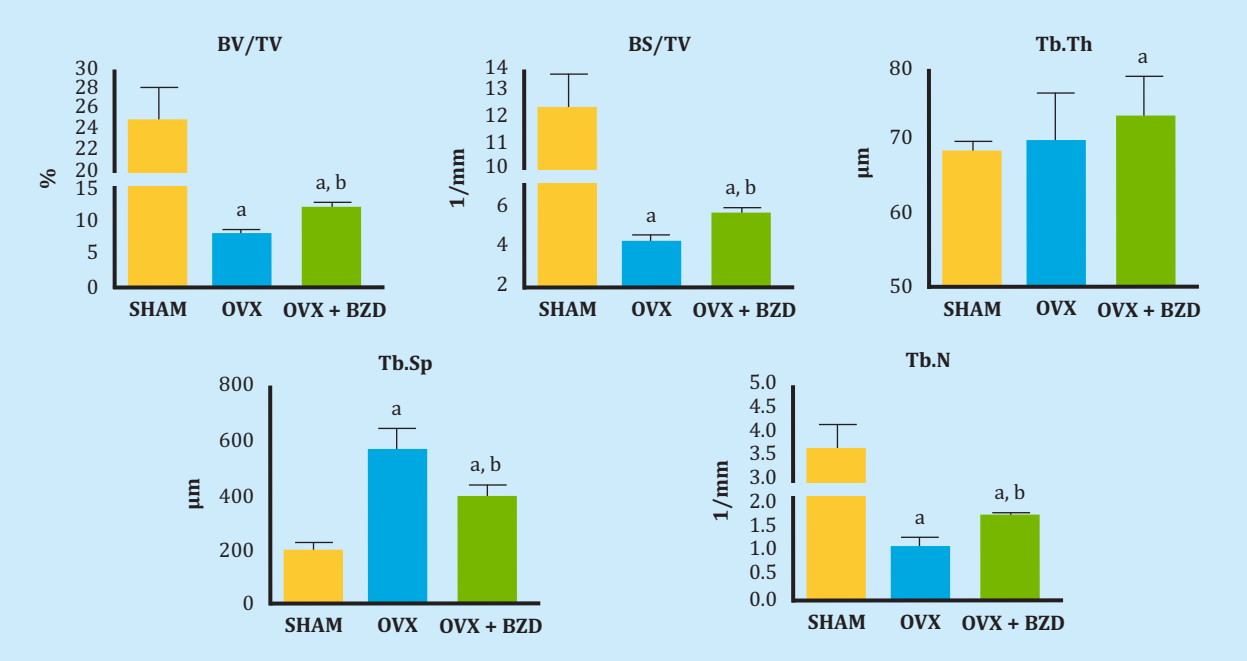
Saito et al.[28] studied ovariectomized female adult monkeys who were administered 0.2 or 0.5 mg/kg bazedoxifene for 18 months. The levels of immature and mature cross-links, BV/TV, and Tb.Th were higher in the group treated with bazedoxifene than in the ovariectomized group. However, the SMI was lower in the group treated with bazedoxifene than in the ovariectomized group. Bazedoxifene treatment prevented the deterioration of immature enzyme cross-link levels, in advanced glycosylation products, and in structural properties such as B/ TV, Tb.Th, and Tb.Pf, which significantly control the bone strength of trabecular tissue.

Regarding biomechanical parameters, we observed in our study that bazedoxifene also exerted a positive action regarding the ovariectomized rats on the maximum deformation to which the femur is subjected when performing a force on it.

Lastly, bazedoxifene produced a positive action on the weight gain experienced by rats after the ovariectomy, being a lesser weight gain than the experienced by ovariectomized rats, although higher than the experienced by the rats in the control group.

Most studies on the effects of bazedoxifene focus on vertebral fractures, such as those previously discussed[14,22-24]. Some authors such as Reginster et al.[29] confirm that bazedoxifene also reduces non-vertebral fracture risk in women with a high risk of suffering os-

Figure 3. Bone volume fraction (BV/TV), bone surface density (BS/TV), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp) and trabecular number (Tb.N) in the 3 groups of rats: SHAM (control), ovariectomized (OVX) and ovariectomized treated with bazedoxifene (OVX + BZD)



BV/TV. a: OVX vs SHAM p<0.01; OVX+BZD vs SHAM, p<0.05, **b**: OVX+BZD vs OVX, p<0.05; **BS/TV. a**: OVX vs SHAM p<0.01; OVX+BZD vs SHAM, p<0.05, **b**: OVX+BZD vs OVX, p<0.05; **Tb.Th. a**: OVX+BZD vs SHAM, p<0.05; **Tb.Sp. a**: OVX vs SHAM p<0.01; OVX+BZD vs SHAM, p<0.05, **b**: OVX+BZD vs OVX, p<0.05; **Tb.N. a**: OVX vs SHAM p<0.01; OVX+BZD vs SHAM, p<0.05, **b**: OVX+BZD vs OVX, p<0.05.

teoporosis. Authors such as Yavropoulou et al.[5] observed an increase in lumbar BMD but not hip BMD after the treatment with bazedoxifene, but, like Reginster[29], they did observe a decrease in the risk of non-vertebral fractures in high-risk postmenopausal women.

Regarding the comparative effect exerted by bazedoxifene and other drugs, in a meta-analysis carried out on 48,000 patients, Liu et al.[30] observed that alendronate and risendronate produced a greater positive effect than bazedoxifene on osteoporosis, but with more side effects. Gatti et al.[31] report that bazedoxifene is as effective as raloxifene in preventing bone loss in women with osteoporosis and in reducing the frequency of new vertebral fractures. Other authors such as Ellis et al.[32] consider that bazedoxifene is comparable to bisphosphonates to prevent vertebral fractures among women with high-risk postmenopausal osteoporosis.

In a study carried out by our group[33], we administered zoledronic acid to ovariectomized rats and we obtained much greater effects on increasing lumbar and femoral BMD on untreated rats than in the case of bazedoxifene. The rats' age conditions and ovariectomy time were totally similar to those in this study, so the results can be compared. Authors like Yavropoulou et al.[5] state that bazedoxifene does not seem to offer significant advantages over other antiresorptive agents, but considering the need for long-term treatments for osteoporosis, it is a drug that has a place in the long-term therapeutic scheme to combat this sickness. Authors such as Gatti et al.[31] suggest that, due to its particular profile, bazedoxifene can be considered as a second-line therapy for women between 65 and 70 years of age where bisphosphonates are contraindicated or poorly tolerated. These authors think that bazedoxifene may also be a first-place therapy in younger postmenopausal women to deal with their menopause and the prevention of osteoporosis, and that it could be prescribed alone or with conjugated estrogens.

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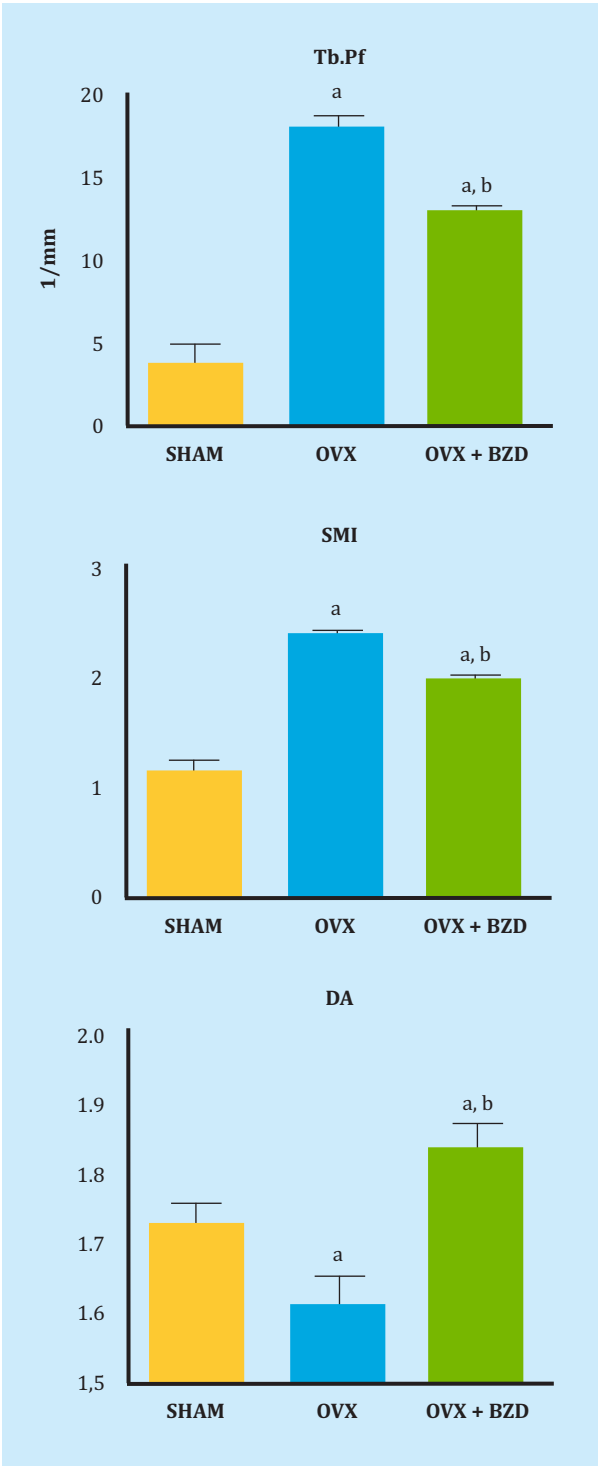
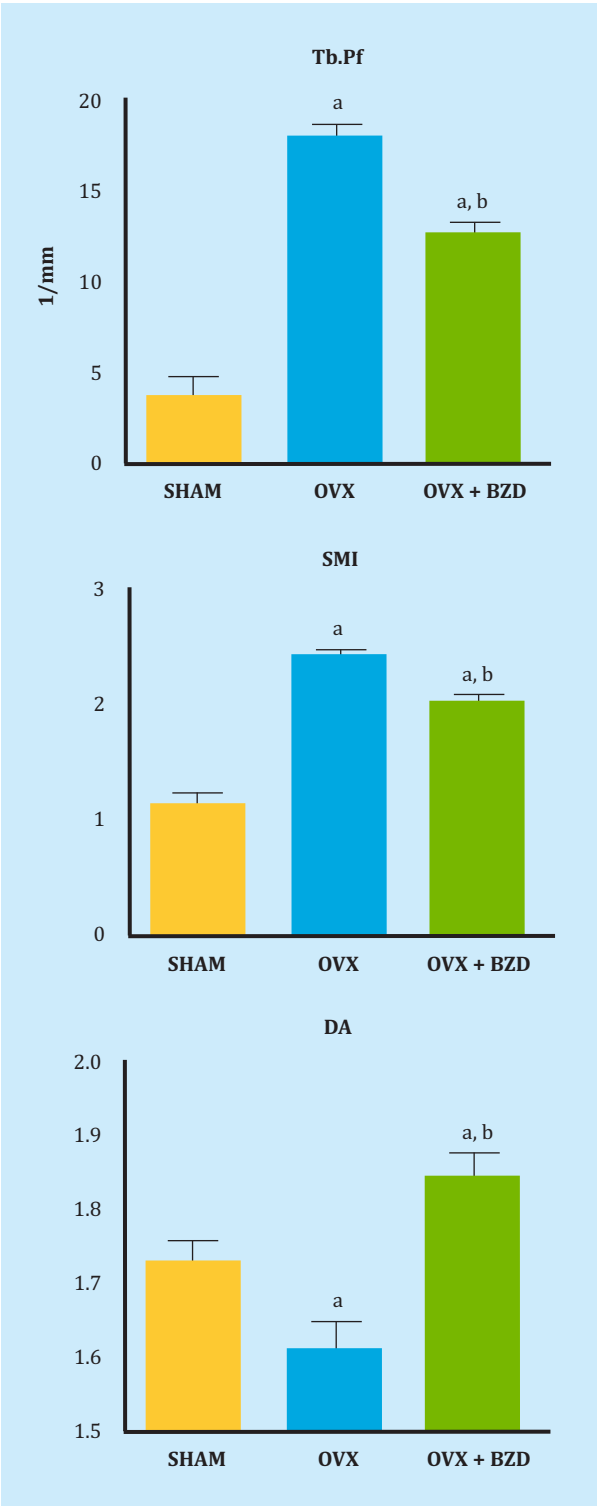
Figure 4. Trabecular pattern factor (Tb.Pf), structural model index (SMI) and degree of anisotropy (DA). In the 3 groups of SHAM rats (control), ovariectomized (OVX) and ovariectomized rats treated with bazedoxifene (OVX + BZD)



Tb.Pf. a: OVX vs SHAM, p<0.01; OVX+BZD vs SHAM, p<0.05, **b**: OVX+BZD vs OVX, p<0.05; **SMI. a**: OVX vs SHAM, p<0.01; OVX+BZD vs SHAM, p<0.05, **b**: OVX+BZD vs OVX, p<0.0; **DA. a**: OVX vs SHAM, p<0.05; OVX+BZD vs SHAM, p<0.01, **b**: OVX+BZD vs OVX, p<0.01.

Figure 5. Bone volume + bone marrow (VIP), cortical bone (Ct.BV) and medullary volume (Md.V) in the cortical in the 3 groups of rats: SHAM (control), ovariectomized (OVX) and ovariectomized rats treated with bazedoxifene (OVX + BZD)



CtBv. a: OVX vs SHAM, p<0.05; OVX+BZD vs SHAM, p<0.05; **Md.V. a**: OVX vs SHAM, p<0.01.

Conflict of interests: Authors declare no conflict of interests.

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Influence of high-concentration hyperbaric oxygen therapy on bone metabolism

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Summary

Objectives: To learn how high concentration in hyperbaric oxygen therapy (HBO) acts on the expression of genes related to bone metabolism in osteoblast cell lines and human trabecular bone.

Material and methods: The differential expression of several genes related to bone metabolism (SOST, RUNX2, MMP14, OPG, HIF-1α and SIRT1) in two human osteoblastic cell lines (Saos and Super-Saos) and in human trabecular bone fragments subjected to one, three or five HBO sessions (90 minutes, 100% oxygen; 2.3 atmospheres). In each experiment, a control that did not receive HBO was used.

Results: We did not find significant differences after HBO in the expression of the genes studied, neither in the cells nor in trabecular bone. Only in the Super-Saos cell line the expression of OPG after 5 sessions of HBO decreased 6 times with respect to that of the control group ($2^{-\Delta Ct}$ Ct of 72; p=0.01).

Conclusions: High concentration oxygen in the hyperbaric chamber (HC) does not seem to influence the expression of genes related to bone metabolism.

Key words: oxygen, hyperbaric chamber, bone, genes.

Introduction

Oxygen is required to produce cellular energy and is involved in numerous processes, such as enzymatic activation, molecular signaling and regulation of gene expression[1]. Also in angiogenesis, the maintenance of hematopoietic stem cells and bone formation[2]. In fact, changes in the partial pressure of oxygen can influence the function of osteoblasts and osteoclasts[3]. In hypoxia, bone formation and mineralization decreases, while resorption increases[4-6]. In the opposite direction, hyperoxia could have a beneficial effect on the bone. Treatment with high concentration of oxygen in the hyperbaric chamber has proven useful in osteomyelitis and osteonecrosis of the jaw caused by radiotherapy or by the use of bisphosphonates[7-9]. HC accelerates osteogenic differentiation of mesenchymal cells and decreases the activation of osteoclasts[10-12].

In this work we wanted to analyze the actions of oxygen at high concentration in HBO on the expression of genes related to bone metabolism in osteoblastic cell lines and human bone[5,6,13,14].

Material and methods

Cell lines

Two osteoblastic cell lines, Saos-2 and Super-Saos, were used. Saos-2, derived from a human osteosarcoma. Super-Saos is a line generated in our laboratory, derived from the previous one and with a high capacity to express the sclerostin gene (SOST)[15]. Both lines were grown in T25 bottles with 5 ml of DMEM culture medium (Dulbeco's modified Eagle culture medium) plus 1% P/S (penicillin-streptomycin) and 1% amphotericin B, and stored in an incubator at 37°C for one week, changing the culture medium every 4 days to cover between 60-80% of the sur-

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face of the bottle. The plates were introduced into the HC (Galeazzi, Italy; 100% oxygen; 2.4 atmospheres) for 90 minutes per session receiving one, three or five consecutive sessions (Figure 1). The same cell line was used as control group subjected to identical culture, transport and handling conditions, but without undergoing HBO.

Figure 1. Hyperbaric chamber

Bone fragments

Trabecular bone fragments extracted from the femoral head of patients with osteoporotic fracture hip replacement surgery were used. After extraction, the bone fragments received a single session of HC (Galeazzi, Italy; 100% oxygen; 2.4 atmospheres) for 90 minutes and subsequently frozen at -70°C. Bone fragments subjected to the same conditions of conservation, culture, transport and handling were used as controls but without receiving HC. This experiment was approved by the Clinical Research Ethics Committee (CEIC) of Cantabria. All patients gave informed consent.

RNA extraction and quantification

24 hours after the last HBO session, RNA was extracted, both in the cell lines and in the bone. In the homogenization process in cell lines, the samples were washed with phosphate buffered saline (PBS) prior to the use of TRIzol®. In the case of bone fragments, TRIzol® was also used, as well as homogenization for 20-30 seconds until the sample was pulverized, and subsequently centrifuged. In both cases the manufacturer's recommendations were followed and the RNA separation, precipitation and resuspension process continued.

Quantitative RT-PCR (polymerase chain reaction with reverse transcriptase) was carried out to detect gene expression: SOST (sclerostin gene), RUNX2 (protein related to transcription factor 2), MMP14 (metalloproteinase 14), HIF-1α (hypoxia-inducible factor), SIRT1 (sirtuin1), OPG (osteoprotegerin) and RANKL (kappa-nuclear nuclear factor receptor activator ligand) using Taqman assays and following the manufacturer's instructions. The threshold cycle (Ct) values were obtained and the data normalized to the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and TBP (TATA box binding protein) using the ΔCt method. To calculate the relative level of mRNA, the formula $2^{-\Delta Ct}$ was used, where ΔCt is the difference between the Ct average of the normalizing genes and the Ct of the gene of interest.

Statistic analysis

We used a non-parametric test, the Wilcoxon test for the comparison of means of two matched groups. Values of $p<0.05$ were considered statistically significant.

Results

HBO effect on RNA expression in the Saos-2 cell line

There were no differences in the expression of genes in the cell line after one, three or five HBO sessions. The differences with respect to the control in $2^{-\Delta Ct}$ Ct after 5 sessions were 0.71 for SOST (p=0.50), 0.89 for SIRT1 (p=0.34), 0.47 for MMP14 (p=0.18), 0.43 for HIF1α (p=0.18), 0.79 for RUNX2 (p=0.65) and 7.91 for OPG (p=0.40) (Figure 2). No RANKL expression was detected.

Effect of HBO on RNA expression in the Super-SaOS cell line

Compared to the control, we found OPG expression decreases 6 times after 5 HBO sessions ($2^{-\Delta Ct}$ Ct, 72 p=0.01). In the rest of the genes there were no differences: $2^{-\Delta Ct}$ Ct, from 1.03 for SOST (p=0.34), 1.46 for SIRT1 (p=0.34), 1.77 for MMP14 (p=0.18), 1.08 for HIF1α (p=0.18), 1.14 for RUNX2 (p=0.18) and 1.24 for RANKL (p=0.31) (Figure 3).

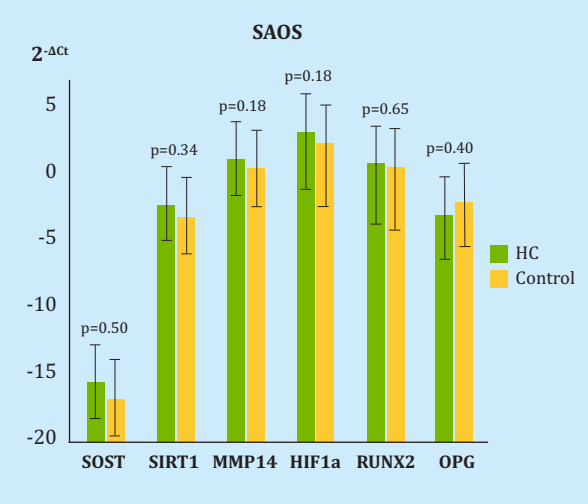
HBO effect on RNA expression in trabecular bone

Nor were there differences in the expression of genes after HBO in bone, only a modest, non-significant increase in the expression of SOST with a $2^{-\Delta Ct}$ Ct change of 5.39 (p=0.48). In the rest of the genes the differences were 0.92 for MMP14 (p=0.58), 1.28 for HIF1α (p=0.81), 0.72 for RUNX2 (p=0.24), 1, 18 for SIRT1 (p=0.42), 1.97 for RANKL (p=0.91) and 3.9 for OPG (p=0.55) (Figure 4).

Conclusions

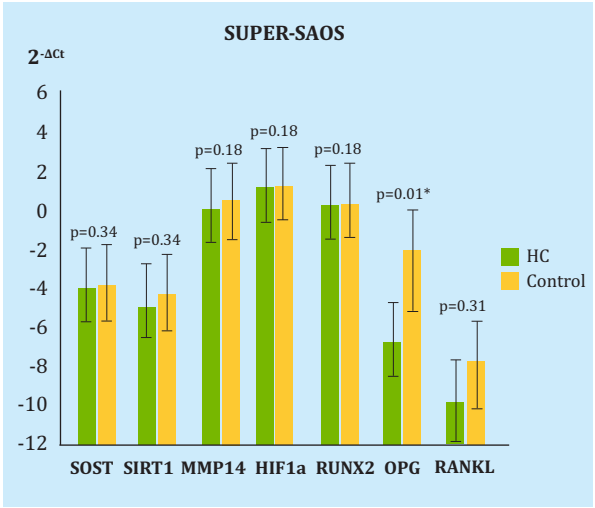
Hyperoxia is considered beneficial for bone by increasing the proliferation and differentiation of osteoblasts[16]. Al Hadi et al.[6] described increased expression of type I collagen and Runx-2 mRNA in osteoblast cell lines (Saos-2) subjected to HBO for 14 days (2.4 ATA, 97% O_2, 90 min/day). HBO also increased the proliferation and differentiation of osteoblasts in human alveolar bone[17]. Hyperoxia also seems to decrease bone resorption. Treatment in HC (100% O_2, 2.4 ATA) reduced the expression of RANK, NFATc1 and Dc-STAMP in the serum of patients and also regulated the expression of the hypoxia inducible factor (HIF-1α)[18]. Other described actions of oxygen at high concentration (100% O_2, 2.4 ATA) are the improve-

Figure 2. Difference in the expression of genes under study and housekeeping in the SAOS cell line after 5 sessions of hyperbaric chamber



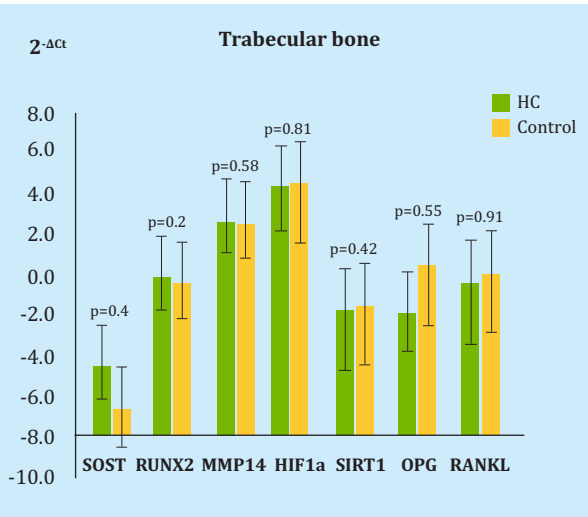
HC: cell group undergoing hyperbaric oxygen therapy; SOST: sclerostin; SIRT1: sirtuin1; MMP14: metalloproteinase 14; HIF-1α: hypoxia inducible factor 1α; RUNX2: protein related to transcription factor 2; OPG: osteoprotegerin.

Figure 3. Difference in the expression of genes under study and housekeeping in the Super-SAOS cell line after 5 sessions of hyperbaric chamber



HC: cell group undergoing hyperbaric oxygen therapy; SOST: sclerostin; SIRT1: sirtuin1; MMP14: metalloproteinase 14; HIF-1α: hypoxia inducible factor 1α; RUNX2: protein related to transcription factor 2; OPG: osteoprotegerin.

Figure 4. Difference in the expression of genes under study and housekeeping in bone after a single hyperbaric oxygen therapy session



HC: cell group undergoing hyperbaric oxygen therapy; SOST: sclerostin; SIRT1: sirtuin1; MMP14: metalloproteinase 14; HIF-1α: hypoxia inducible factor 1α; RUNX2: protein related to transcription factor 2; OPG: osteoprotegerin.

ment in angiogenesis, increased vascularization of the aspirated iliac crest of mice[19], greater cell proliferation[20] or acceleration in the healing of open femoral fractures in experimental animals[21]. However, most of these works have been carried out in animal models and human studies are scarce. In patients with avascular necrosis of the femoral head, serum OPG levels increased after HBO (5.61±1.99 pmol/L at baseline, 7.90±1.9 pmol/L after 15 sessions, 8.97±2.07 pmol/L after 30 sessions; $p<0.05$), without changes in RANKL[22] levels. After HBO (2.5 ATA, 100% O_2 for 90 min/day), osteogenic differentiation of bone marrow mesenchymal cells was also improved in treated patients, with an up-regulation in Wnt3a, b-catenin and Runx2 and descending GSK-3b, compared to those who did not receive it[12]. These same authors also described an increase in bone morphogenetic protein (BMP2) and Osterix in treated patients[12].

In our study, we did not find that oxygen at high concentration in HC influences the expression of different genes related to bone metabolism (SOST, SIRT1, MMP14, HIF1a, RUNX2, OPG and RNAKL). However, we would highlight that we found a slight tendency, not significant, to the increase in the expression of SOST in the bone undergoing treatment. We know that oxygen tension influences the regulation of SOST and that in hypoxia (1% oxygen tension) osteoblasts and osteocytes express low levels of SOST and sclerostin[23], perhaps this is due to a lower expression of prolyl hydroxylase (PHD2) since it has been seen that deletion of PHD2 in osteocytes causes a lower production of sclerostin dependent on SIRT1[14]. This pathway could elucidate our understanding of the pathophysiological mechanism through which, and in the opposite direction, an oxygen-rich environment could increase the expression of SOST and sclerostin. In fact, our group has found a 25% increase in serum sclerostin levels in 12 patients undergoing HBO treatment. However, other works are contradictory[13].

In conclusion, it does not appear that hyperoxia in HC influences the expression of genes related to bone metabolism, although we believe that more studies are needed to broaden our knowledge of the actions of oxygen in bone.

Conflict of interests: Authors declare no conflict of interests.

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Assessment of bone mass density in the surgical indication. New tool

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The proximal humerus fracture represents 5 to 8% of all fractures and is twice as frequent in women as in men. These fractures occur mainly in patients with bone fragility. They are among the most frequent along with hip and distal radius fractures in patients older than 65 years[1-4], thus presenting a multidisciplinary challenge. Since proximal humerus fractures have been considered fragility fractures, the role of general and local bone mineral density is increasingly gaining attention in the literature[5-8].

The influence of local bone mineral density on the functional outcome of the treatment of proximal humerus fractures is controversial. Classically, it has not been sufficiently addressed in the literature. However, the most recent studies show that osteoporosis can negatively affect surgical treatment and subsequent consolidation of fractures of the proximal humerus. That is why bone quality should be part of the preoperative evaluation[6,9].

Barnett and Nordin first reported the determination of cortical thickness as a predictor of skeletal mineralization in 1960[10]. Since then, measurements of the cortical thickness of the femoral shaft and metacarpals have been widely used to estimate osteoporotic changes in bone. However, cortical thickness of the distal humerus has been shown to be an even more reliable predictor for detecting generalized osteoporosis than that of femoral or metacarpal cortical osteoporosis[11].

The use of a simple measurement to determine the bone quality of the proximal humerus could help in making surgical decisions, allowing the indication of the most appropriate technique. For example, it may be possible to predict the safety of screw fixation in bone[11].

The Tingart measurement[11] is the most frequently used method to measure bone quality in AP x-rays of the shoulder. However, in patients presenting a proximal humerus fracture, the reference points required for the Tingart measurement are often involved in the fracture. In addition, measurement errors must be corrected by x-ray magnification, and there is not always a reference to perform it.

Recently, another index that relates cortical thickness to bone quality is increasing in the literature: the deltoid tuberosity index (DTI). The necessary measurements for it are made immediately above the upper end of the deltoid tuberosity. At that level, the outer cortical edges become parallel; the DTI is equal to the relationship between the external cortical diameter and the internal endostal diameter. When this ratio is less than 1.4, there will be low bone mineral density in the proximal humerus[9].

Unlike what happens with the Tingart index, the location of the precise measurements to calculate the DTI are far from the fracture lines. Furthermore, the deltoid tuberosity generally appears well defined in AP x rays, possibly due to the antalgic position that is normally adopted, with the arm in internal rotation[9].

In their study, Spross et al.[9] found that the correlation between radiographic measurements and local bone mineral density was strong for the DTI and moderate for the Tingart measurement. Likewise, inter-observer reproducibility was higher in DTI.

Thus, we consider DTI to be a reliable, simple, and applicable tool to assess local bone quality in the proximal humerus. Furthermore, its use has better clinical applicability in patients with proximal humerus fractures than the Tingart index, since sometimes the fracture lines reach the reference points of this measurement.

In this way, Spross et al.[12] have generated a comprehensive algorithm as a treatment guide for FHP, where the demands and biology of the patient are prioritized, being a useful tool for decision-making, achieving a low rate of complications and revisions.

We thus believe that a comprehensive patient assessment, with its different facets, weighing each one in its proper measure, will bring us closer to reality. Hence, considering this global vision of the patient, not limiting ourselves solely and exclusively to the fracture, will make the difference between being good or achieving excellence.

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