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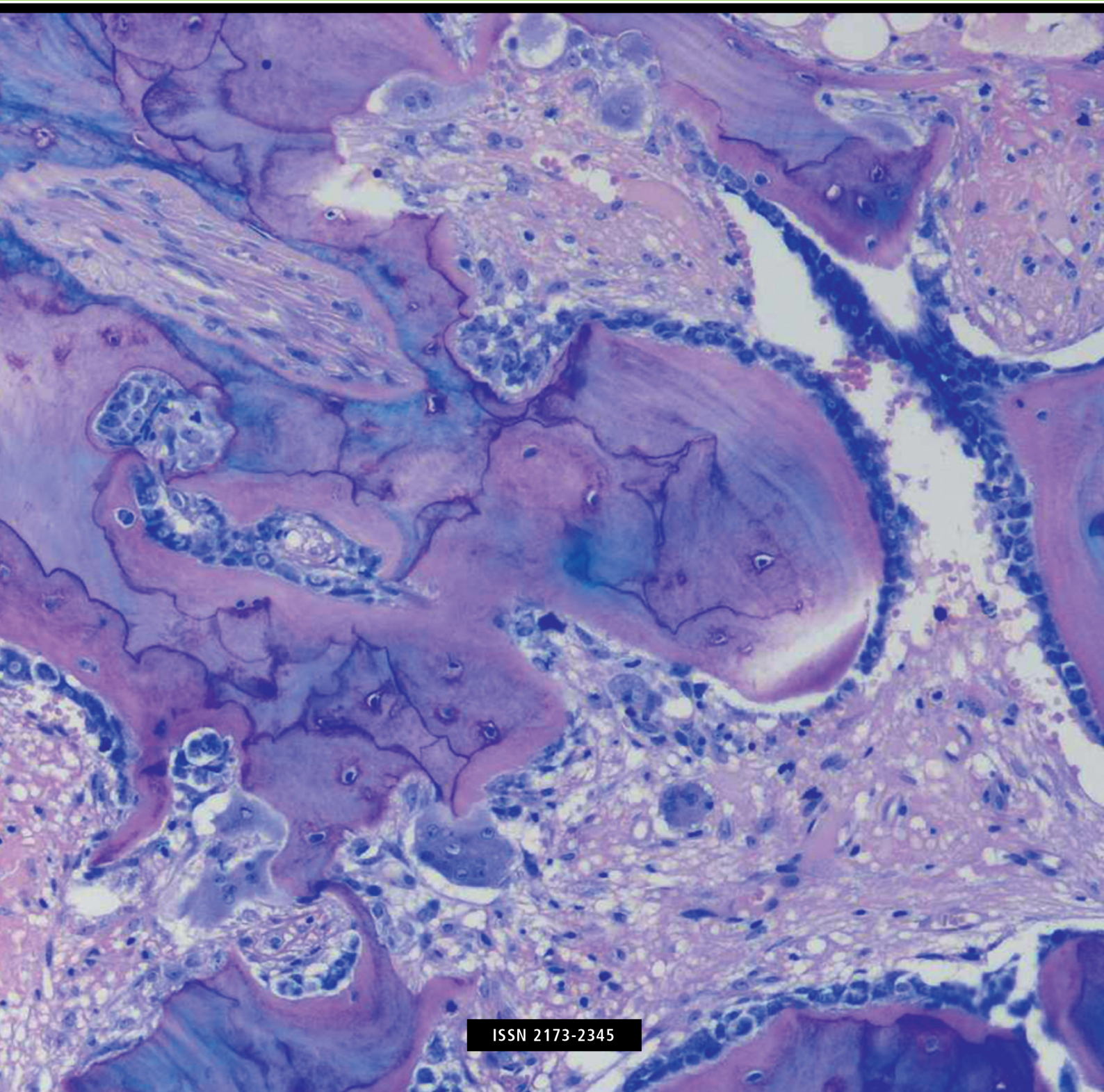

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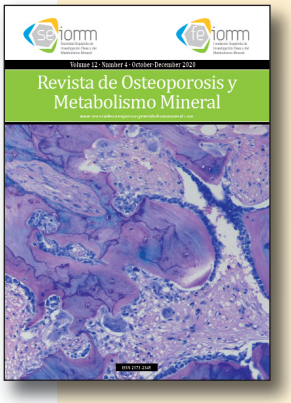

Our cover: Mosaic pagetoid trabeculae, lined by numerous osteoblasts. There are few osteoclasts, less active in appearance.
Author: Dr. Manuel Diaz Curiel. Bone Metabolic Diseases Consultant. Jiménez Díaz Foundation University Hospital. Madrid (Spain).

Summary

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Are femoral bone mass measurements symmetrical?

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This issue of the journal offers an interesting article on possible differences in femur densitometry related to the dominance of the upper extremities between left and right handed[1].

Dual-energy X-ray absorptiometry (DXA) is based on the measurement of areal bone mineral density centimeter (BMD, g/cm^2) in the proximal femur and lumbar spine. Conditions such as osteoarthritis or osteophytic calcifications influence spinal BMD and confer a great value to femoral measurement. Since the DXA technique began being used on the hips, the presumption that there may be a minimal bilateral asymmetry between the proximal femurs has been maintained, but with no clinical relevance. Several research groups have studied this question. It has not been established whether there are systematic differences between the BMD of both hips, and in order to answer the questions: is the bone density in one of the femurs similar to same in the opposite side? which of them to choose?

In this sense, the forearm example is paradigmatic, for it is a region of interest consigned to an alternative sector and yet used in situations where measurements in conventional regions are not reliable. Due to the regarded differences in the BMD of the dominant and non-dominant forearms, the measurement of the BMD of the non-dominant forearm to reduce the variance is recommended.

The answer to "are the left and right proximal femurs symmetrical?" is important, since the result of the measurement in the chosen hip affects the fracture risk estimation in the subject. In their quest to find ideal hip replacements and improving surgical techniques, orthopaedic surgeons show special interest towards possible anatomical differences.

Research on anatomical variations of the proximal femur has revealed differences regarding gender and ethnicity between femurs. Differences in femoral dimensions and compensations between men and women have been identified[2-5]. Other studies have found differences in some morphological characteristics in femurs from European and Asian populations, specifically in the diameter of the femoral head, femoral displacement, and diameter of the shaft[6]. Different properties such as bone mineral density, mechanical resistance, cortical thickness, angles or length of the femur have also been studied[7-11]. The assessment of possible differences in 160 paired femurs from both sides of corpses showed absolutely no differences regarding any femoral measurement exceeding 1.5 mm[9]. The percentage of asymmetry did not exceed 4% for all anthropometric measurements and they found no link between absolute differences and the percentage of asymmetry, gender and/or ethnicity. Age or weight did also have nothing to do with absolute differences or percentage of asymmetry. This study, as well as other publications, supports the assumption of a high degree of symmetry in the left and right proximal femurs despite their shape and the shape of the body[12,13]. Symmetry is generally independent of demographic data and overall dimensions of the proximal femur.

By applying the DXA method with different technological approaches, other groups have assessed the variations in femoral BMD and geometric characteristics such as the length of the hip axis (HAL) between left / right femur and have found a high correlation (r=0.81-0.96) in the relevant regions of interest. They did not detect significant differences between both sides, so even though there is a dominant forearm, there does not appear to be a dominant hip. The authors of these studies[1,14,15] have concluded that the measurement of a single femur is usually sufficient for the clinical evaluation of BMD and/or the length of the hip axis.

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

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Comparison of the femur proximal extremity's densitometric values in young and healthy study participants: left-handed vs. right-handed

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Summary

Objetive: Physical activity is a key factor for bone mineral density. Left-handed people exercise more left limbs than right-handed do. The objective of this study was to determine whether left-handed participants have higher values of BMD in the left lower limbs (proximal femur) and right-handed subjects have them higher in the right lower ones.
Material and methods: Cross-sectional observational study performed on young and healthy men and women who do not practice any sport activity, and who were divided into two groups according to their laterality, established by the Edinburgh Handedness Inventory. The bone mineral density in the lumbar spine and the proximal extremity of both femurs was measured in all of the participants using a Hologic QDR 4500 Discovery® densitometer.
Results: From the 122 study participants, 62 were right-handed and 60 were left-handed. Statistically significant differences were not perceived among the participants, nor age-related, or in male-female proportion, body mass index or according to the subjects' lifestyle: alcohol consumption, tobacco use and physical activity practiced during leisure time. Left and right-handed participants showed similar values for bone mineral density in the spine and in all the anatomical regions measured (femoral neck, total hip, trochanter and intertrochanter) in the right and left femurs. However, lower BMD values were obtained in all the measured locations of the left femur, compared to the same measurements in the right femur (these differences being statistically significant) when considered all the participants as a whole or when grouping them according to their laterality.
Conclusions: Differences of BMD in the measured locations of both right and left-handed participants were not observed. However, the BMD values in the left side were significantly lower in all subjects, regardless of their laterality.

Key words: bone mineral density, laterality, lef-handed, right-handed, prevalence.

Introduction

Dual-energy X-ray absorptiometry, commonly known as bone densitometry[1], is a technic broadly used in daily clinical practice and is considered the gold standard to estimate the bone mineral density (BMD)[1-4]. When performing a densitometry, the values obtained, usually in the lumbar spine and in the proximal extremity of the femur, are compared with the reference values for the population of each country, so the T-score and Z-score values can be calculated[3-5]. By consensus, the World Health Organization recommended the osteoporosis densitometric diagnosis to be carried out in the presence of a T-score value lower than -2.5 of the typical deviation of the peak BMD[2]. Although this criterion has been a topic for controversy, it has also become a world reference that has allowed the homogenisation of the randomized trials, among other advantages[1-6].

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On the other hand, the beneficial effect of the mechanical stimulus on the bone is well known. Physical exercises that entail a significant load are one of the best ways to increase BMD, as it has been seen in those athletes who present an asymmetric load on one of the limbs, something present for example in tennis or soccer players, who show significant BMD differences in the dominant limb[7-15].

Approximately 10% of the population is left-handed[16]. At the beginning of the twentieth century, several studies comparing the BMD in left and right-handed non-athletes were carried out and presented diverse results[17-19]. However, we have not found recent studies on this matter presenting more conclusive results. This is the reason why we have undertaken the present work, as we come from the hypothesis that the left lower limb in left-handed subjects may suffer a higher load during daily physical activity, therefore they would show higher levels of BMD; at the same time, we should see exactly the opposite in right-handed subjects: higher levels of BMD in right femur.

Material and methods

Lifestyles. Questionnaire

This is a cross-sectional observational study, including a total of 122 volunteers, male and female students of the Faculty of Health Science of the University of Las Palmas de Gran Canaria (ULPGC) without known pathologies. All the subjects who suffered from any pathology that could affect or which treatment could affect the bone mineral metabolism were excluded. We identified the participants' lifestyles by means of a questionnaire, validated beforehand[20].

Alcohol consumption was drawn up by means of the AUDIT survey (alcohol used disorder identification test), considering high-risk alcohol consumption those intakes equal or higher than 35 SDU (standard drink unit) in male participants per week and 21 SDU in female participants per week [21]. An SDU in Spain is established at 10 g, which is equal to the average alcohol content of a wine or beer intake, and of half an intake of a distilled drink[22].

The physical activity was estimated by applying the short form of the IPAQ questionnaire (The International Physical Activity Questionnaire – Short form, IPAQ-SF)[23]. Sedentary behaviour meant being sat down for more than six hours per day.

Anthropometric determinations

All the subjects underwent a physical examination, including height and weight measurements (wearing a light outfit), from which we obtained their body mass index or Quetelet index (BMI) defined as weight in kilograms divided by height in meters squared, overweight being estimated when that index was equal or higher than 25 kg/m^2 and obesity when it was equal or higher than 30 kg/m^2 [24].

Laterality

The participants filled the Edinburgh Handedness Inventory[25], which consists of fifteen items in its wider version, being this one the version we used in our study, available via the link: https://www.brainmapping.org/shared/Edinburgh.php.

Each of the subjects obtained a score ranging from -100 to +100. According to this scale, the participants obtaining negative scores ranging from -20 to -100 were identified as left-handed, and those obtaining positive scores ranging from +20 to +100 were considered right-handed. We excluded from our study any ambidextrous subjects in order to make comparisons between both groups, right-handed and left-handed, exclusively.

Consent. Ethics

All the participants were explained the objectives of the study in detail and they all signed an informed consent form. The protocol was previously approved by the Clinical Trial Committee of the Insular University-Materno Infantil Hospital Complex.

Bone densitometry

The BMD was measured by means of a Dual-energy X-ray absorptiometry (DXA) using a Hologic QDR 4500 Discovery® densitometer (Hologic Inc. Waltham, USA). This densitometer uses an Xray tube (XR) and the source of radiation and energy pulses alternately at 70 Kvp and 140 Kvp, being transmitted by a tube with a 2 mA peak. In the multi-center study performed by the Working Group on Osteoporosis (WGO), a variation coefficient between 0.75%±0.16 with a range between 0.6 and 1.13% was established for the densitometry carried out using this same equipment[26]. All the determinations were made by the same operator, so there are no interobserver differences. All the measurements were carried out in the lumbar spine, in AP projection of the L2-L4 vertebrae. Subsequently, the measurements in both proximal extremities of the femur were carried out in the following anatomical regions: femoral neck, total hip, trochanter and intertrochanter.

The theoretical mean value and the standard deviation of each age group were obtained from the values considered normal for the population of the Canary Islands[27].

Statistic analysis

Categorical variables were expressed as frequencies and percentages, and continuous variables as means and standard deviations when the variables followed a normal distribution, or as medians with their interquartile ranges (IQR = 25th –75th percentiles) when the distribution drifted apart from normality. The percentages were compared using the chi-square test (χ^2) and Fisher's exact test. The means were compared by applying the Student t test and the medians were compared by applying the Wilcoxon test for independent data.

Results

122 volunteer subjects participated in the study. Figure 1 shows the participant selection process.

Table 1 shows the baseline characteristics of the study participants, 60 left-handed and 62 right-handed. Two ambidextrous subjects were excluded for the reasons indicated in material and methods. It is a young population with an average age of 24 years (24.3 years vs. 23.7 years), with a notable predominance of females, since approximately 73% of the participants were women (73.3% in the left-handed group vs. 72.6% in the right-handed group). The body mass index (BMI) was similar in both groups, the same being within normality. Most of the participants were non-smokers and drank in moderation, with no statistically significant differences between left-handed and right-handed, with similar prevalences. Likewise, most of the participants were sedentary (more than 40% in both groups), with no statistically significant differences between them.

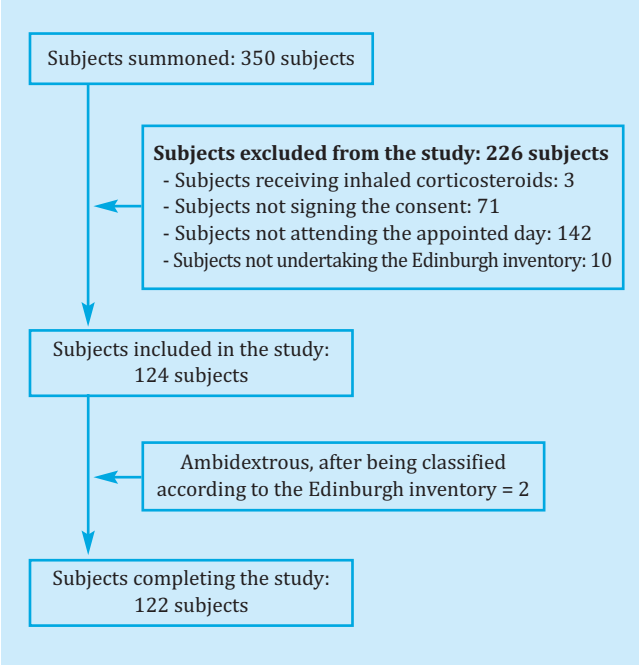
Table 2 shows the BMD values measured by DXA between left-handed and right-handed; no statistically significant differences were obtained in any of the anatomical regions where the measurement was carried out, both in the lumbar spine and in the proximal extremity of the femur (femoral neck, total hip, trochanter and intertrochanter) of the two extremities, right and left. Laterality did not show differences in BMD. However, it was observed that the BMD showed higher values in the right femur vs. the left one in all the regions and in both groups (positive [right-left] differences). When comparing these differences between the two groups, left-handed participants showed a greater difference than right-handed participants, although it was only significant in the intertrochanteric region (p=0.203).

Given this finding, the differences between right and left densitometric values were analyzed to see if they were statistically significant. Table 3 shows the densitometric results obtained in all the participants, without grouping them by laterality. When comparing the densitometric values of the right femur with those of the left femur, as when comparing them in the groups separately, we obtained that in all anatomical regions (femoral neck, total hip, trochanter and intertrochanter) the BMD values on the right side were higher than on the left side, with statistically significant differences in all the anatomical regions.

Table 4 shows the results of this comparison in the groups studied, left and right-handed, obtaining that the differences were significant in all the regions except in the femoral neck, both in the left-handed and the right-handed groups.

Figure 2 shows the correlation obtained in the BMD determinations in the proximal femur between both sides, right and left, in left and right-handed participants, and in each anatomical region.

Figure 1. Flow chart showing the study participants selection process



Discussion

Although the influence of laterality on BMD in athletes has been shown in various studies[7-15], it has not been so in subjects who do not perform intense physical activity. As it was the case in other similar studies, we have studied a healthy and young population, so there could be no influences, derived from age, menopause, diseases or therapies, affecting their BMD. Although determining the laterality of an individual is sometimes difficult, the Edinburgh Handedness Inventory[25] has allowed us to draw an objective classification, in contrast to what has been observed in some of the published studies on left and right-handed people, in which the participant's self-assessment is accepted, almost always only taking in consideration the hand they use to write. In fact, the application of this inventory forced us to exclude 2 participants who, seeing themselves as left-handed, were actually ambidextrous.

When classifying those selected participants into left-handed and right-handed, we obtained two very homogeneous groups in terms of age, proportion of men and women, BMI and lifestyles that can influence bone, such as the alcohol consumption[28,29], tobacco use[30,31] and physical exercise[32,33]. Given that they were all healthy young people, without medication that could affect the bone mineral metabolism, we could consider laterality as a determining factor of the differences observed in BMD between both groups.

Our working hypothesis was that left-handed participants should show higher BMD values in all anatomical regions of the left lower limb where it was measured, while right-handed participants should have higher values in the right proximal femur, due to a higher load in it. We established this hypothesis as we observed that in some studies in the literature, carried out in athletes, when an asymmetric load occurred in one limb, the densitometric values obtained were higher in the dominant limb, both in the upper limbs (in the case of the tennis players)[7-10] as in the lower limbs (in the case of the soccer players)[11-13]. This fact was also observed in sedentary subjects[34]. We also start from the hypothesis that differences in lumbar spine BMD should not be observed between the two groups, as it is a structure of medium location without influence in laterality, under normal conditions. Since what we wanted to assess was the load effect on the bone, we did not consider the BMD measurement in the forearm.

Similar studies carried out previously showed contradictory results: in 2001, Dane et al.[17] studied the BMD in both femurs of 124 right-handed and 23 left-handed students, showing that in right-handed men the BMD was higher in the left femur, while in women there were no significant differences. They also found that the BMD in the non-dominant femur was higher than in the dominant one, both in left-handed as a whole (right femur) and in right-handed (left femur) in all the anatomical regions, except in the trochanter among right-handed, which was the opposite. These results were significant among the right-handed, but not among the left-handed (except in the trochanter, with the opposite result).

In the study by Gümüştekin et al.[18], carried out in 2004 also in students (32 right-handed and 26 left-handed), similar results were obtained: right-handed participants, men and women together, and separated by sex, showed higher BMD in the left femur in all the anatomical regions, the significant difference being the total me-

Table 1. Baseline characteristics and lifestyles of the study participants

	Left-handed N = 60	Right-handed N = 62	P value
Age, years	24.3 ± 8.3	23.7 ± 8.5	0.675
Female gender number (%)	44 (73.3)	45 (72.6)	0.925
Body mass index, kg/m^2	23.5 ± 3.7	22.7 ± 3.1	0.192
Tobacco use			0.593
Smokers (%)	7 (11.7)	11 (17.7)	
Non-smokers (%)	48 (80.0)	45 (72.6)	
Former smokers (%)	5 (8.3)	6 (9.7)	
Alcohol consumption			0.292
Risk regular drinkers (%)	2 (3.3)	0 (0.0)	
Abstainees (%)	19 (31.7)	16 (25.8)	
Moderate drinkers (%)	39 (65.0)	46 (74.2)	
Regular physical exercise			0.905
Intense activity (%)	23 (38.3)	26 (41.9)	
Moderate activity (%)	11 (18.3)	10 (16.1)	
Sedentary (%)	26 (43.3)	26 (41.9)	

Data are expressed as means ± standard deviation and number (percentage).

Table 2. Densitometric values obtained in the lumbar spine and in the proximal limb of both femurs in left and right-handed participants. Net values and after subtracting the values on the right side from those on the left

	Left-handed N = 60	Right-handed N = 62	P value
L2-L4, g/cm^2	1.047 ± 0.105	1.044 ± 0.131	0.883
Femoral neck, g/cm^2			
Right	0.889 ± 0.134	0.889 ± 0.122	0.998
Left	0.876 ± 0.120	0.879 ± 0.127	0.863
Right - left	0.013 ± 0.053	0.009 ± 0.042	0.651
Total hip, g/cm^2			
Right	0.999 ± 0.130	0.996 ± 0.120	0.882
Left	0.984 ± 0.130	0.982 ± 0.120	0.933
Right - left	0.015 ± 0.037	0.013 ± 0.035	0.826
Trochanter, g/cm^2			
Right	0.756 ± 0.119	0.752 ± 0.105	0.853
Left	0.733 ± 0.108	0.733 ± 0.098	0.988
Right - left	0.023 ± 0.046	0.019 ± 0.034	0.634
Intertrochanter g/cm^2			
Right	1.156 ± 0.152	1.146 ± 0.143	0.685
Left	1.128 ± 0.146	1.131 ± 0.143	0.903
Right - left	0.029 ± 0.070	0.014 ± 0.050	0.203

Data are expressed as means ± standard deviation.

Table 3. Bone mineral density in the different anatomical regions of the proximal limb of the femur, globally comparing both sides in all study participants

	Right side	Left side	P value
Femoral neck g/cm^2	0.889 ± 0.127	0.878 ± 0.123	0.011
Total hip g/cm^2	0.997 ± 0.124	0.983 ± 0.124	<0.001
Trochanter g/cm^2	0.754 ± 0.111	0.733 ± 0.103	<0.001
Intertrochanter g/cm^2	1.151 ± 0.147	1.130 ± 0.144	<0.001

Data are expressed as mean ± standard deviation.

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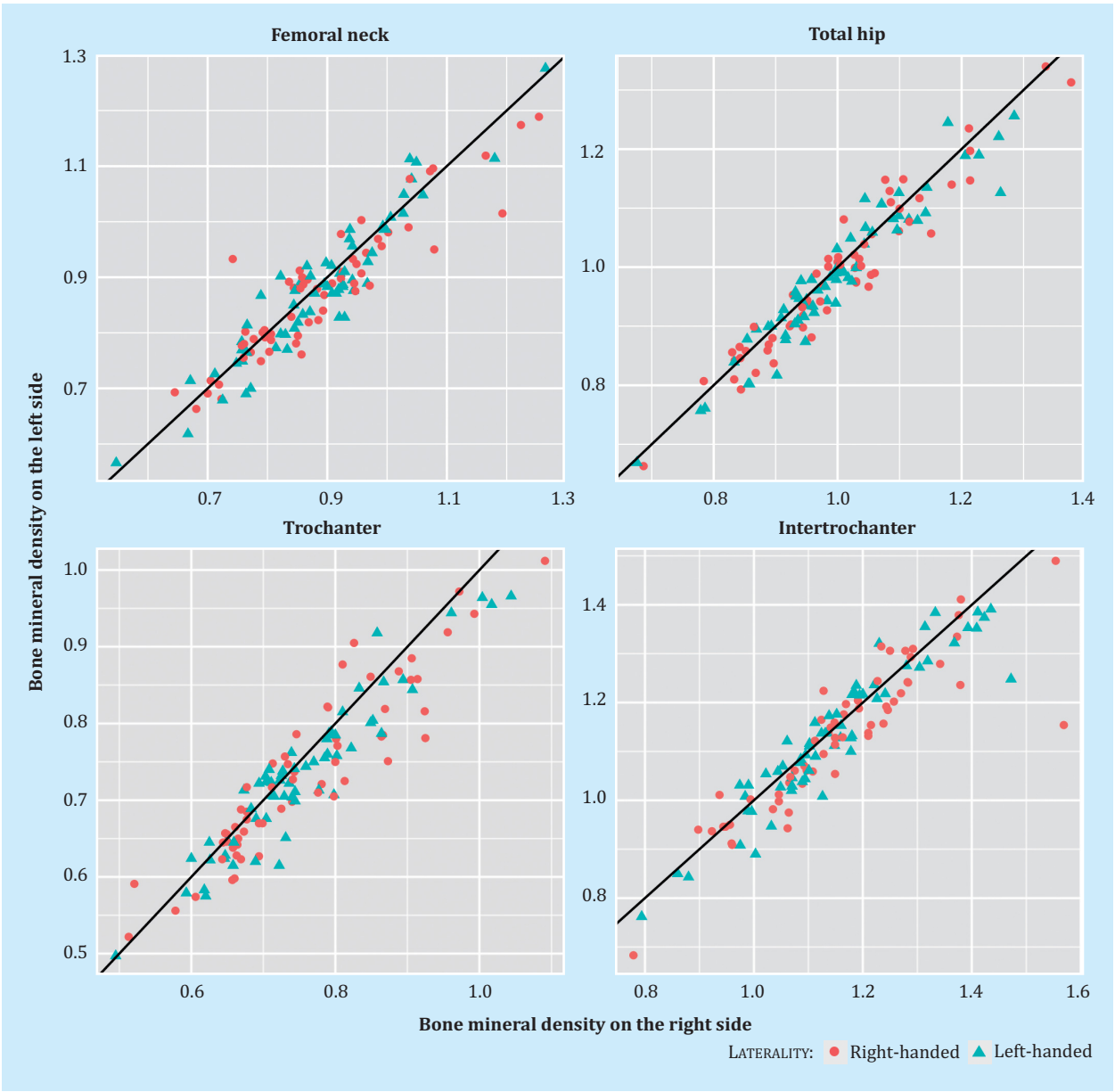
Table 4. Comparison between the bone mineral density values (g/cm²) in the different anatomical regions of the left proximal femur with those of the right in each group studied, left and right-handed participants

	Left-handed			Right-handed		
	Right side	Left side	P value	Right side	Left side	P value
Femoral neck	0.889 ± 0.134	0.876 ± 0.120	0.060	0.889 ± 0.122	0.879 ± 0.127	0.094
Total hip	0.999 ± 0.130	0.984 ± 0.130	0.003	0.996 ± 0.120	0.982 ± 0.120	0.004
Trochanter	0.756 ± 0.119	0.733 ± 0.108	<0.001	0.752 ± 0.105	0.733 ± 0.098	<0.001
Intertrochanter	1.156 ± 0.152	1.128 ± 0.146	0.002	1.146 ± 0.143	1.131 ± 0.143	0.026

Data are expressed as mean ± standard deviation.

Figure 2. Correlation of the BMD values obtained in the different anatomical regions of both proximal extremities of the femur in both groups, left and right-handed participants



asurements (and intertrochanteric measurements in men); while left-handed people showed higher BMD in all the anatomical regions of the right femur (although only significant in the intertrochanteric region), except in Ward's zone in the group, and femoral neck and Ward's zone in men, which was the opposite (higher BMD on the dominant side). Like Dane et al., they found that BMD in the non-dominant femur was higher, both in left- and right-handed, although the differences were not significant in general.

In 2009 Sahin et al.[19] published a study carried out with 113 students, 66 men and 47 women (66 right-handed and 47 left-handed in total) in which it was reported that the BMD of both femurs (right and left) in the right-handed participants was higher than in the left-handed ones and in a significant way (except in Ward's area of both femurs). These authors did not assess the differences between the BMD values between the left and right femur of each group.

In our study, we did not consider analysing the results by sex, because, when working with small subgroups, the differences would not be conclusive. Contrary to what was reported in the aforementioned studies, we did not obtain statistically significant differences between left and right-handed participants in the densitometric values obtained for any of the anatomical regions we measured in the proximal extremity of the right and left femurs. Obviously, we did not get them in the lumbar spine either. This indicates that, in the population we studied, laterality did not influence the BMD values and, nor is there difference in the load of both lower limbs, or this load is not enough to affect the BMD. Our volunteers led mostly sedentary lives, and this may justify the above explanation. In articles by Dane[17], Gümüştekin[18] and Sahin[19], no assessment is made of the physical exercise of the subjects studied, so we do not know if it could have influenced the difference in BMD found.

However, we were struck by the higher BMD found in all the anatomical regions of the right femur compared to those of the left, both in right-handed (which would be expected) and left-handed separately, as well as in the total number of participants, and in some cases, of a significant importance. These results were contrary to other authors' reported findings[17,18]. Our results were more homogeneous than in these other studies, in the sense that all the anatomical regions were the same (not so in the studies by Dane and Gümüştekin[17,18]). On the other hand, we found no general explanation for this higher BMD in the proximal right femur; other studies published in larger populations of age obtain opposite results; thus, Rao et al. conducted a study on 131 osteoporotic Caucasian women, finding that the BMD of the left hip was significantly higher than that of the right in all the anatomical regions measured, not being able to determine the influence of laterality due to the low number of left-handed women (only 7, 5%)[35]. In contrast, Bonnick et al. carried out BMD measurements in both proximal femurs in 198 women with an age ranging from 16 to 73 years old, without considering their laterality, and only found significant differences in the trochanteric region, the means of the differences being generally low (neck = 0.7%, Ward's zone = 0.2%, and trochanter = 1.9%), although they found individual differences as high as 22%[36].

Despite the differences found, and as in the rest of the studies reviewed, our results show a correlation between the BMD values of the left and right proximal femurs; this correlation is widely accepted, and, in fact, the position documents of the International Society of Clinical Densitometry (ISCD)[1] show that, for the measurement of BMD in the proximal femur either of the 2 extremities can be used. However, if we accept that the fundamental validity of this test is to identify subjects with low BMD, given the inverse relationship between the densitometric values and the risk of fracture, having estimated that each decrease in one standard deviation doubles the risk of fracture[4,6] and in view of our results, in which the left proximal femur presents significantly lower BMD values than the right, it might be advisable to perform the measurements on the left femur.

A limitation of our study was the small sample size. This is observed in almost all studies assessing laterality, because the proportion of left-handed subjects in the general population is low (approximately 10%)[37].

In conclusion, according to our results, laterality in the participants who do not perform a sport or physical activity that entails a significant load on the dominant limb does not favourably affect their BMD. Furthermore, the lower BMD observed in the measurements of the left proximal femur compared to the right one, regardless of laterality, lead us to suggest that it would be more appropriate to carry out the densitometry on the left femur if we want greater diagnostic sensitivity.

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

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Fracture risk predictors of a postmenopausal female population by binary statistical procedure CART

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Summary

Objetive: The main consequences of osteoporosis are fragility fractures, associated with high morbimortality. The prediction of these fractures can help identify the most-at-risk population and implement preventive measures. The aim of this study was to assess the usefulness of multiple factors in their prevention, comparing the bone mineral density (BMD), the calculation of absolute risk of fracture using the tool FRAX® in the presence and absence of BMD, and the clinical data.

Material and method: An eight-year-duration longitudinal study was conducted on a postmenopausal female population, with and without osteoporosis. All of them were taken a standardised clinical history, spinal and hip BMD, and FRAX with and without BMD. Eight years later we identified the existent fractures. In addition to a parametric and non-parametric statistic in SPSS 21.1, we used the classification and regression tree (CART) method to assess possible interactions among fracture risk factors.

Results: We studied 276 postmenopausal patients whose average age at the beginning of the study was 61.08±8.43 years-old and had a body mass index (BMI) of 25.67±4.04. 56.5% of the patients (n=156) were diagnosed with osteoporosis before the beginning of our study, and all of them were treated. After eight years of follow-up, 72 patients (24.6%) suffered a fracture and 17 patients (6.2%) also suffered a second one. The results of the CART analysis showed that the main risk factor to suffer an osteoporotic fracture after 8 years of following up is having preceding fractures. Having a femoral neck BMD lower than 0.67 was the main risk factor among those with a previous fracture.

Conclusions: The use of a binary statistical procedure (CART) on a cohort of patients allow us identify those most at risk of fractures, according to clinical parameters and simple additional tests, in order to establish more effective therapeutic measures.

Key words: osteoporosis, fracture, FRAX, bone mineral density, CART analysis.

Introduction

The osteoporosis is an illness linked to a high morbimortality that increases as the population grows older. It has been defined as a systemic skeletal disease characterized by a deterioration of bone micro-architecture and a decrease of bone tissue, with a consequent increase in bone fragility and a higher susceptibility to fracture[1]. It is a clinically silent disease that is not manifested by other signs but for its complications, fractures.

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The main consequences of osteoporosis are fragility fractures that can appear in different locations, though they typically happen on the vertebrae, distal radius and proximal extremity of the femur[2,3]. They are fractures with a high economic cost and are associated with a higher morbimortality, specifically those on the vertebrae and the proximal femur. Hip fracture mortality, the most serious manifestation of osteoporosis, is 8% during the first month after the fracture (acute mortality). It rises to 30% after a year[4]. Furthermore, the recovery of patients who do not pass away is poor. Only 30% of patients suffering a hip fracture return to the baseline situation[5]. The vertebral fracture shows a higher incidence than the hip fracture. While the hip fracture shows a yearly incidence of 1.3-1.9 cases/1,000 inhabitants/year, the incidence of vertebral fractures is 13.6/1,000 inhabitants/year in males and 29.3/1,000 inhabitants/year in females[2]. Although its mortality is lower than that of hip fracture, it is not despicable, especially in patients also presenting a respiratory disease[6,7]. Therefore treatments are designed to prevent its appearance through adequate therapeutic measures. In order to establish the most appropriate treatment it is necessary to dispose of stand-alone diagnostic factors that help identify the every patient's individual risk through additional tests or risk scales.

Bone mineral density (BMD) has demonstrated an ability to predict fracture risk and also to prove the efficacy against fractures of different treatments[8]. This has been proven by a meta-analysis, although its usefulness in individual patients is less measurable[9]. Moreover, there are a high number of fractures whose BMD levels are in the range of osteopenia[10].

Due to the current difficulty in some countries to carry out densitometries, different clinical procedures have been developed to establish fractures risks and indication of densitometry[11]. These procedures have not been clearly implemented owing to the development of the FRAX® tool, which is a risk scale sponsored by the World Health Organization consisting of a very simple-to-use computer tool whose risk has been adjusted by country. Its purpose is to identify the risk of fracture in men and women between 40 to 90 years[12]. However, it also poses problems by not including important fracture risk factors such as falls, the lack of definition of osteoporosis secondary causes or the corticosteroid dosing, the underestimation of osteoporotic major fracture risk in some populations and its invalidity on treated osteoporosis patients. On the other hand, simple clinical data reportedly have prediction ability comparable to that of the FRAX[10].

The objective of this study, then, was to assess the fracture prediction ability of BMD, of the FRAX with or without BMD and of clinical data in an osteoporosis-treated female population and in a non-osteoporotic female population showing risk factors of the disease over an eight-year follow-up, by using the classification and regression tree method (CART). Its ultimate goal is to identify those patients with the highest risk of osteoporotic fracture.

Patients and methods

Patients

A retrospective and longitudinal study was conducted in postmenopausal women with suspected osteoporosis or with osteoporosis diagnosis. Inclusion criteria were: having amenorrhea the preceding twelve months, and osteoporotic diagnostic or diagnostic suspicion according the clinical criteria established by the National Osteoporosis Foundation Clinician's Guide to Prevention and Treatment of Osteoporosis[13]. Exclusion criteria were: the absence of osteoporosis risk factors, the lack of monitoring data and not signing the written informed consent. All women were taken a standard clinical history including demographic data, lifestyle-related factors and prior diseases. Their height and weight were measured in order to get their body mass index (weight –kg-/height^2 –m-). Baseline data were collected in 2011. All patients were calculated the FRAX with and without BMD. All their clinical histories were checked again in 2019. Clinical fractures were identified through the reports from the traumatology and emergency wards. In case of any doubt, X-rays were checked. Lateral spine X-rays were assessed in order to identify incidental fractures by applying the Genant criteria[14].

Bone densitometry

To establish BMD, a densitometer DXA Prodigy® (GE Healthcare, Madison, Wisconsin, USA) was used following the manufacturer's recommendations. BMD was carried out on the lumbar spine (L1-L4), femoral neck and total hip. The T-score was appraised using the values of normality for the Spanish population.

Statistical analysis

Continuous variables are expressed as median±standard deviation (SD), while categorical variables are expressed as absolute (n) and relative (%) frequencies. We used Chi-square test to compare the categorical variables. The Kolmogorov-Smirnov test was used to analyse the distribution of the variables.

The analysis of variance test was used to get parametric variables (ANOVA) while non-parametric variables were determined by the Mann-Whitney U test (two groups) or the Kruskal-Wallis test (more than two groups).

CART analysis was used to asses possible interactions among fracture risk factors statistically linked to having suffered a fracture after eight years of follow-up. CART analysis is a binary partitioning method which provides a graphic structure similar to a decision tree[15]. This allows the identification of subgroups of subjects with a higher risk of suffering an osteoporotic fracture. The pool of patients featuring the entire sample is classified in groups based on a dependent factor (in this case: patients who have suffered fractured vs. patients who have not suffered fracture). During the procedure, all possible independent factors (or variables) are examined and the factor that is more closely connected with regard to the dependent variable is selected. Then, two new groups are created (nodes). This partitioning process is repeated for each node and stops when there is no statistical association between a dependant variable and the rest of independent variables, or when the size of the group sample is small. The Bonferroni correction was applied to the CART analysis.

P values less than 0.05 were considered significant. All the analyses were carried out by means of the statistical package SPSS 22.0 (SPSS, Chicago, Illinois, USA).

Ethical aspects

The protocol complies with the Declaration of Helsinki (2008) by the World Medical Association. It was approved by the Ethics Committee of Río Hortega University Hospital (Valladolid, Spain) and is in line with Spain's

data protection law (LO 15/1999) and its specifications (RD 1720/2007). All patients who agreed to participate in the study signed a written consent.

Results

Our work included the study and follow-up of 276 postmenopausal patients whose mean age at the start of the study was 61.08±8.43 years. The BMI was 25.67±4.04. Almost all the patients were Caucasian (n=274, 99.6%), only one patient was of South American ethnicity (0.4%). Regarding gynecological data, the age at menarche was 13.03±1.46 years and the age at menopause was 47.99±5.75 years. The patients had a mean of 2.07±1.3 children. 56.5% of the patients (n=156) were diagnosed with osteoporosis before the start of our study, and all of them were treated. After eight years of follow-up, 72 patients (24.6%) had suffered a fracture and 17 (6.2%) had also suffered a second fracture; 61 patients (22.1%) had suffered a fracture being over 55 years of age. The data for the global population are shown in table 1.

There were 16 (8.6%) patients treated with thiazides, 32 (17.1%) with serotonin receptor inhibitors, 1 (0.5%) with androgen inhibitors, 8 (4.3%) with beta-blockers, 20 (10.7%) with thyroid hormones, 74 (27%) with antiresorptive agents, 4 (1.5%) with hormone replacement therapy, 11 (4%) with anabolic therapy, 25 (9%) with corticosteroids and 10 (3.6%) with strontium ranelate. Table 2 shows the characteristics of the included patients divided into two groups: patients with fractures occurred during the eight years of follow-up and patients without fractures.

The results of the CART analysis showed that the main risk factor for suffering an osteoporotic fracture after 8 years of follow-up was having suffered previous fractures. Among patients who had suffered a previous fracture, having a femoral neck BMD less than 0.67 was the main risk factor. Among the patients who had not suffered previous fractures, the main risk factors were BMI and having a FRAX without BMD (major osteoporotic fracture) greater than 9.30 (Figure 1). Figure 2 shows the results of the CART analysis in patients diagnosed with osteoporosis. The main risk factors for suffering a fracture after 8 years of follow-up were having suffered previous fractures and presenting a BMD of the femoral neck less than 0.663. Among the patients who had not suffered previous fractures, the main risk factor was being over 67 years of age (Figure 2). Finally, the results of the CART analysis in patients not diagnosed with osteoporosis showed that the main risk factors for suffering an osteoporotic fracture were being over 55 years of age and having previously suffered the associated non-bone-related disease, comorbidity (Figure 3).

Discussion

In our study, the data that allow us to pin-point patients who have suffered an osteoporotic fracture during follow-up are the existence of a previous fracture, age, and FRAX with or without BMD. This last data loses part of its value in the CART analysis, which is why it is probably a marker of disease severity rather than a predictor in our series. In the CART analysis, which takes into consideration the factors that may influence the subsequent appearance of fractures, we witnessed that the key element for the total population is the existence of a previous fragility fracture supplemented with a BMD lower than 0.67 in the femoral neck, while in the absence of

Table 1. General characteristics of the population included in this study

Age (years)	61.08 ± 8.43
Age of menarche (years)	13.03 ± 1.46
Age of menopause (years)	47.99 ± 5.75
Number of children	2.07 ± 1.20
BMI (kg/m²)	25.67 ± 4.04
FRAX without BMD (major osteoporotic fracture)	2.17 ± 3.83
FRAX without BMD (hip fracture)	2.17 ± 3.83
FRAX with BMD (major osteoporotic fracture)	12.49 ± 9.11
FRAX with BMD (hip fracture)	3.15 ± 4.04
FRAX with BMD (hip fracture), high risk	71 (27.6%)
Family history of fracture	42 (15.2%)
Family history of osteoporosis	104 (37.8%)
Tobacco	88 (30.0%)
Alcohol	3 (1.1%)
Preceding fractures	87 (37.8%)
Pathological history (excluding bone-related)	62 (19.7%)
Spine BMD, g/cm²	0.860 ± 0.130
Femoral neck BMD, g/cm²	0.834 ± 0.138
Total hip BMD, g/cm²	0.860 ± 0.130
Osteoporosis	156 (56.2%)

BMI: body mass index; BMD: bone mineral density. Data expressed as means ± standard deviation and absolute number (percentage).

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Table 2. Clinical characteristics of the patients included in this study, comparing those who have suffered fragility fractures to those who have not

	Fracture after eight years of follow-up.		
	No (n = 215)	Yes (n = 61)	P value
Age (years)	57.74 ± 7.40	60.96 ± 5.69	0.039
Age of menarche (years)	12.91 ± 1.52	13.03 ± 1.50	0.711
Age of menopause (years)	48.45 ± 5.50	49.26 ± 5.04	0.501
Number of children	2.05 ± 1.23	1.96 ± 1.19	0.736
BMI (kg/m²)	25.30 ± 4.29	27.21 ± 4.08	0.043
FRAX without BMD (major osteoporotic fracture)	3.98 ± 3.06	5.01 ± 3.52	0.14
FRAX without BMD (hip fracture)	1.05 ± 1.45	1.32 ± 1.79	0.414
FRAX without BMD (hip fracture), high risk	4 (4.3%)	2 (7.4%)	0.514
Family history of fracture	11 (11.8%)	4 (14.8%)	0.68
Family history of osteoporosis	33 (35.9%)	8 (29.6%)	0.549
Tobaco	37 (39.8%)	11 (40.7%)	0.929
Alcohol	1 (1.1%)	1 (3.7%)	0.348
Preceding fractures	17 (18.3%)	9 (33.3%)	0.095
Pathological history (excluding bone-related)	29 (31.2%)	4 (14.8%)	0.094
Spine BMD, g/cm²	1.04 ± 0.14	1.0 2± 0.19	0.526
Femoral neck BMD, g/cm²	0.87 ± 0.15	0.86 ± 0.10	0.735
Total hip BMD, g/cm²	0.90 ± 0.13	0.91 ± 0.10	0.697

BMI: body mass index; BMD: bone mineral density. Data expressed as means ± standard deviation and absolute number (percentage).

fractures, a BMI of 28 or lower and a FRAX for a major osteoporotic fracture above 9.3 would be the determining elements. In patients diagnosed with osteoporosis, under active treatment, the data are similar to those described for the general population except that, in those cases without a previous fracture, FRAX did not play any role in determining that 67 years of age is the distinguishing data. This is logical given that FRAX is of no use in treated osteoporotic patients. In patients without osteoporosis, the factor that determines the appearance of fractures is an age of over 55 years and the associated non-bone-related pathology, comorbidity.

The importance of secondary prevention is based on the fact that prior fractures constitutes the first determinant in the appearance of posterior fractures[16,17]. A high percentage of men and women are considered at high risk, which has been determined by a previous fracture and not diagnosed or treated[18]. This occurs for any fragility fracture location, being especially significant for vertebral and hip fracture. In the first case, in addition to the fracture itself, its severity according to Genant's classification and the number of fractures increase the risk[19]. Hip fracture is the most serious complication of osteoporosis, not only because of its ability to predict subsequent risk, but also because of its high morbimortality. Furthermore, in this case, BMD is a strong and consistent predictor of posterior fracture[20]. New studies carried out in real clinical practice to assess the efficacy of different treatments have shown that the increase in BMD is a determining factor in its efficacy reducing fractures[21]. In our study, the existence of a BMD in the femoral neck below 0.660 was a strong predictor consistent with these data. Another important factor in the appearance of new fractures is the time factor. This is where the concept of imminent risk of fracture has arisen, which is greater in the first two years after the fracture[22,23]. In a study carried out in a group that included 377,561 older women, the greatest risk of fracture was found to occur in the following 5 years, and especially frequent in the first two. These authors found that the determining factors were age, the location of the fracture and the associated bone disease[24]. In our study, this imminent risk and the location of the posterior fractures have not been determined. However, in patients not diagnosed with osteoporosis, age and associated pathology were present, coinciding with the data from this large series.

Age was another important risk factor in our CART analysis, especially in patients without prior osteoporosis and in patients with osteoporosis but without prior fracture. Numerous published studies have confirmed this fact. Ensrud et al. [25], in a group of 6,652 women and with a 10-year follow-up, concluded that models based on age and BMD, or on age and fractures, predicted risk in a similar way to FRAX. Similar data were obtained by Bolland et al.[26] when comparing two scales, FRAX and Garvan, with age and BMD. In the Glow study, with 19,586 female participants over 60 years of age, it was observed that a model based on age and previous fractures was superior to FRAX and Q-Fracture without BMD[27].

Another relevant fact of our study is the small role of FRAX in our CART analysis. We can see that the FRAX, both with BMD and without BMD, makes differences

Figure 1. CART analysis (Classification and regression tree) in order to study the main risk factors associated with the risk of suffering an osteoporotic fracture in all the patients included in our study

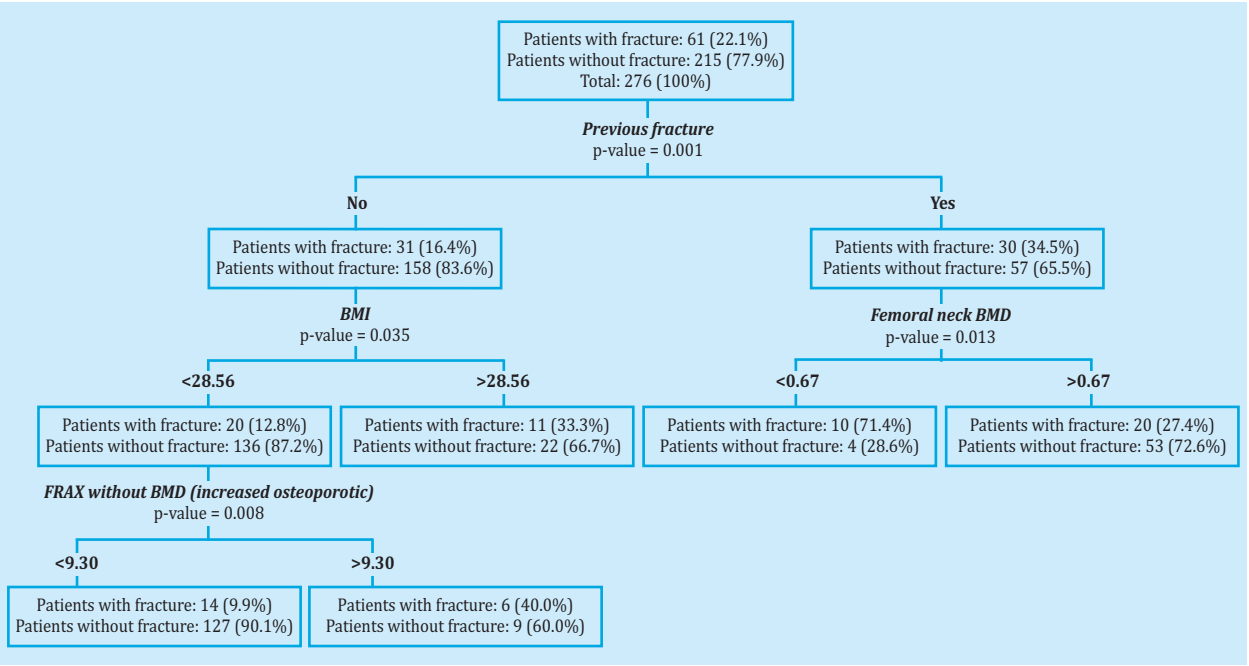


Figure 2. CART analysis (Classification and regression tree) in order to study the main risk factors associated with the risk of suffering an osteoporotic fracture in those patients diagnosed with osteoporosis

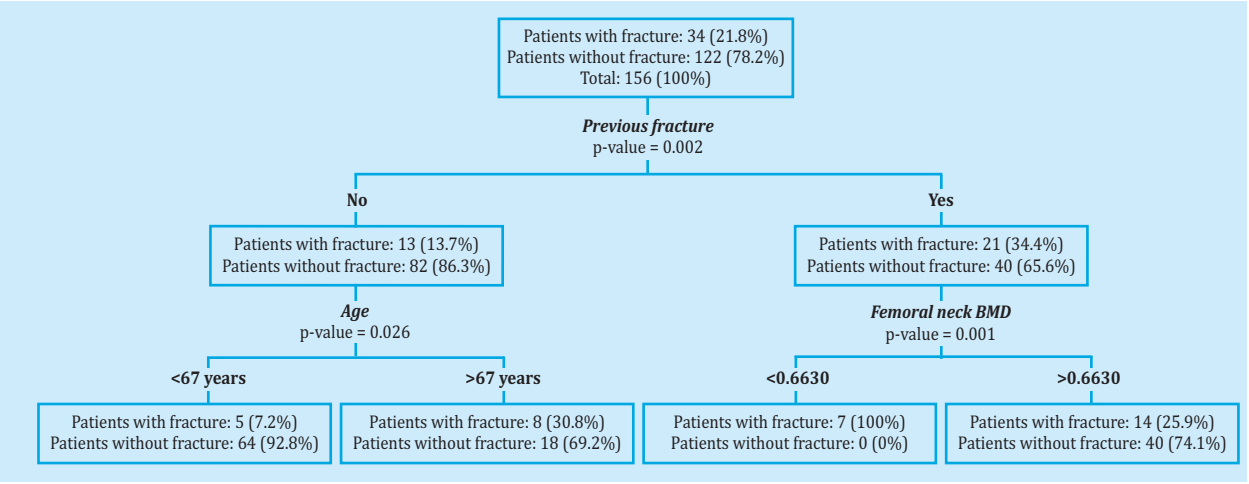
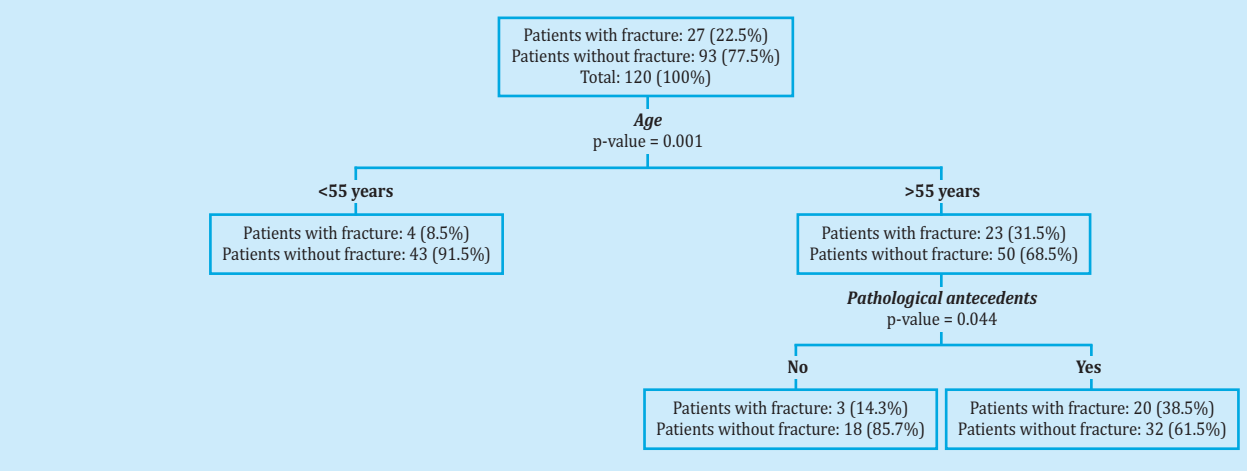


Figure 3. CART analysis (Classification and regression tree) in order to study the main risk factors associated with the risk of suffering an osteoporotic fracture in osteoporosis-free patients



between the patients who do and do not fracture, but when conducting a statistical analysis based on algorithms, its value disappears. Only when analysing the total population, a FRAX for major osteoporotic fracture without BMD, higher than 9.3, has a role in patients without fracture. This FRAX value coincides with the FRAX thresholds established by Azagra et al.[28] in their analysis of the Fridex cohort. These authors divided the patients into three groups, considering those with a FRAX greater than 10 as high risk. This may be due to several facts. In the Spanish population, FRAX underestimates the risk of major osteoporotic fracture. Several cohorts have tried to validate it by analyzing the differences between predicted and observed fractures[29-31]. The results can be considered acceptable in predicting the risk of hip fracture, but not for that of a major osteoporotic fracture, probably due to the lack of robust epidemiological data for this type of fracture. Comparing the FRAX with other simpler tests, including only age, has not shown a greater predictive ability[32]. Another fact of our study is that osteoporotic patients were receiving active treatment, therefore, it was not possible to validate FRAX in their cases.

Another noteworthy fact in non-osteoporotic patients is the importance of non-bone-related pathological records, comorbidity in the appearance of fractures. Unfortunately, there is no single approved index to assess fragility in clinical practice[33]. Some used indices have been associated with an increased risk of fractures[34] and falls[35]. The Glow cohort and the CaMos cohort used different indices to assess the relationship between fragility and fractures, but both include many of the parameters assessed in our study. The variables of the Glow fragility fracture index included 15 items on comorbidity, 12 on basic activities of daily living (similar to the Barthel index), 6 items on signs and symptoms (fullness of life, energy, exhaustion, fatigue, non-intentional self-assessed pain/discomfort, weight loss). The fragility index used in the CaMos cohort included 30 items, 13 referring to pathology, 5 to functional aspects (vision, hearing, gait, manual dexterity/use of tools and cognition) and 12 to general health and daily activities. However, the Charlson index was not associated with the risk of fracture, although some studies, such as the SIDIAP registry, which included 186,171 men, found that a Charlson index ≥3 was linked to an increased risk of hip fracture[36]. The newest finding in this study is the use of the CART statistical methodology to establish the risk of posterior fracture in a heterogeneous population that included osteoporotic patients under active treatment and non-osteoporotic patients.

In conclusion, the use of a binary statistical procedure (CART) in a cohort of patients allows us to identify patients with a higher risk of fractures based on clinical parameters and complementary tests that are simple to carry out and establish more effective therapeutic measures.

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

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Evaluation of bone mineral density and 3D-Shaper parameters in congenital hypophosphatasia of the adult

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Summary

Objetive: To evaluate bone mineral density (BMD) and 3D-Shaper parameters at the proximal femur (FP) level in adults with genetically confirmed hypophosphatasia (HPP) and to compare them in those subjects with and without fractures.
Material and methods: Cross-sectional analysis of densitometric data and bone architecture from the baseline visit of a longitudinal study in which patients with HPP were included. A densitometric study (Lunar Prodigy, GE iDXA) was carried out in FP using 3D-Shaper software (version 2.7. Galgo Medical).
Results: 33 adults with HPP with heterozygous mutations were included. 63.6% (21/33) were women (42.9% postmenopausal), and 8 of the men (66.6%) were older than 50 years. The mean age was 50.56±15.08 years, 30.3% (10/33) had previous traumatic fractures and 15.2% (5/33) presented stress fractures. The prevalence of osteoporosis in CF was 11.8% (2/17) and of osteopenia, 82.4% (14/17). In premenopausal women and young men, low bone mass was detected for age in 12.5% (2/16). When comparing subjects with and without stress fractures, as well as traumatic ones, there were no differences in BMD. The 3D-Shaper showed a decrease in cortical thickness (mm) in patients with stress fractures [1.8 (1.77-1.89)] compared to subjects without them [1.94 (1.87-2.03, p=0.03)] and compared to those with traumatic fractures [1.97 (1.88-2.04), p=0.03].
Conclusions: These data reflect a discrete densitometric impact in milder forms of the adult. Bone architecture studies could be of interest in determining patients susceptible to stress fractures.

Key words: osteoporosis, hypophosphatasia, bone densitometry, 3D-DXA.

Introduction

Hypophosphatasia (HPP) is a rare metabolic disease characterized by low enzymatic activity of non-tissue-specific alkaline phosphatase (TNSALP), which causes an accumulation of its natural substrates: inorganic pyrophosphate (PPi), pyridoxal-5'-phosphate (PLP) and phosphoethanolamine (PEA)[1]. PPi acts as a potent inhibitor of hydroxyapatite crystal formation and its high extracellular levels can induce skeletal alterations, such as decreased bone mineralization[2,3]. In general, the more severe forms are associated with earlier symptoms and diagnosis, even perinatal, while the milder forms often present later in childhood or adulthood[4]. The importance of an early diagnosis lies in the potential severity of the disease and the alteration of the quality of life, as well as in the possible iatrogenesis derived from a wrong diagnosis and treatment[5]. Previous studies have analyzed the symptoms that characterize adult HPP, which usually shows a wide range of clinical manifestations, sometimes nonspecific, such as the presence of musculoskeletal pain, weakness, dental pathology or early loss of teeth, and the presence of of recurrent stress fractures and pseudofractures[6,7]. In a pediatric age cohort, the analysis of bone mineral density (BMD) in these patients has detected low values in the most severe cases[8].

However, the limited evidence available in adults with HPP has shown a normal or slightly decreased BMD[7,9-12], from which it is deduced that bone densitometry may not adequately predict the risk of fracture[7]. Therefore, the objective of this study is to evaluate the BMD in the proximal femur (PF) and carry out a volumetric analysis of the cortical and trabecular bone of this region, as well as cortical thickness using 3D-Shaper in subjects with HPP, and compare these parameters between subjects with and without a history of fractures.

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Material and methods

Study population and design First, a search was carried out in the biochemical database of our tertiary hospital and associated outpatient clinics in which 383,353 patients with alkaline phosphatase (APH) determinations were located, of which 231,805 were adults with at least two measurements. Of these, 427 showed persistent hypophosphatasemia (≥2 determinations less than or equal to 35 IU/L and none greater than 45 IU/L; normal range: 46-116 IU/L). Subsequently, their medical records were reviewed, and 31 subjects were excluded due to underlying secondary causes of hypophosphatasemia[13] and 13 due to the impossibility of telephone contact. A total of 383 subjects met the selection criteria and were contacted, of which 85 signed the informed consent for the performance of a genetic test to detect variants in the ALPL gene. 39 (46%) patients with pathogenic or probably pathogenic mutations were detected and offered follow-up in our consultations.

This paper presents the cross-sectional analysis of the baseline densitometric and bone architecture data of 33 adults subsequently included in a prospective observational longitudinal study carried out at Hospital La Paz (Madrid). Details related to the recruitment process have been reported in a previous publication by our group[14]. The study has been approved by the Ethics and Research Committee of Hospital La Paz. Informed consent has been obtained from the patients.

Methods

They were collected using a protocolized questionnaire and risk factors for osteoporosis were analyzed, including smoking, alcohol intake (≥30 g/day), sun exposure (≥10 minutes/day), exercise practice, dietary calcium intake from dairy products (1 serving = 1 glass of milk = 2 yogurts = 1 serving of cheese (40-50 mg) = 200 mg/calcium), personal history of fracture and its etiology, as well as family history of hip fracture.

A densitometric study (Lunar Prodigy, General Electric Medical Systems iDXA) was performed in each of the subjects for the analysis of BMD in FP (neck, trochanter, total hip and femoral shaft). The presence of osteoporosis has been defined according to the WHO criteria[15]. On the other hand, the 3D-Shaper software (version 2.7, Galgo Medical) was used to evaluate the volumetric density of the cortical and trabecular bone of the FP. The software uses a 3D statistical model of the FP and adjusts it on the densitometric image, to achieve a personalized 3D model of the shape and distribution of the bone BMD. The measures provided by the software include volumetric bone mineral density (BMD) of the cortical, trabecular, and integral compartments, cortical superficial bone mineral density (BMD), and cortical thickness. Additional information on the methodology implemented in the software and its validation can be found in previous works[16].

Statistic analysis

For the description of the sample, the mean and standard deviation (SD) or median and interquartile range (IQR) were calculated for the quantitative variables, as well as the absolute number and the relative percentages for the qualitative variables in each group. Comparisons between groups were made using the U-Mann-Whitney statistical test. All analyzes were performed using the SPSS version 23.0 statistical package for Windows.

Results

Demographic, clinical, densitometric and 3D-Shaper data of subjects with HPP

Thirty-three adults with HPP were included, all of whom had pathogenic or probably pathogenic variants in heterozygosity. 63.6% of the patients (21/33) were women (42.9% postmenopausal), and 8/12 of the men were older than 50 years (66.6%). The mean age was 50.56±15.08 years; BMI, 26.31±4.39 kg/m^2, and mean alkaline phosphatase, 25.2±6.53 IU/L. 12.1% (4/33) had a family history of hip fracture. The total number of fractures was 16: 30.3% (10/33) had a personal history of fractures of traumatic etiology (3 in the metatarsals, 4 in the hand bones, 2 in the elbow and one in the clavicle) and 15.2% (5/33), previous stress fractures (three patients in one metatarsal, one patient in two metatarsals and one patient, atypical femoral shaft fracture that meets the criteria of the American Society for Bone and Mineral Research (ASBMR)[17], without previous exposure to diphosphonates. No patient had fragility fractures.

The demographic characteristics and the rest of the risk factors for osteoporosis are described in table 1. The prevalence of osteoporosis in the femoral neck was 11.8% (2/17), and osteopenia was detected in 82.4% (14/17) of patients. In premenopausal women and men under 50 years of age, low bone mass was observed for the age range in 12.5% (2/16) of patients. The rest did not show alterations in the densitometric study of the femoral neck (FN). The mean BMD in the femoral neck of the patients with osteoporosis was 0.73±0.01 and the T-score was -2.65±0.7. In patients with osteopenia, the mean BMD in this region was 0.86±0.05 g/cm^2 and the T-score was -1.3±0.43. In subjects with low bone mass for their age range, the mean BMD was 0.67±0.07 g/cm^2 and the Z-score was -2.4±0.6.

In relation to stress fractures, two postmenopausal patients had both femoral diaphysis and metatarsal fractures, and both had bone densitometry in the osteopenic range, while the three premenopausal patients with metatarsal fractures had normal densitometry. Among the patients with traumatic fracture, five were men (four aged >50 years). One of them suffered a traumatic elbow fracture and presented osteoporosis in CF, while the other three suffered metatarsal, elbow and scaphoid fractures, and presented osteopenia. The patient under 50 years old had a traumatic fracture in a metacarpal and normal bone densitometry.

The median BMD at the CF level was 0.876 (0.83-0.92) g/cm^2; at the level of the femoral shaft, 1.123 (1.03-1.21) g/cm^2; 0.759 (0.693-0.82) g/cm^2 in the trochanteric region, and 0.931 (0.89-1) g/cm^2 in the total hip. The Z-score value at the CF level was -0.29 (-1.13-0.25) and in total hip, -0.06 (-0.79-0.39). In the 3D-Shaper analysis, a total cortical BMD of 813.45 (759.12-862.26) mg/cm^3 was observed, a trabecular BMD of 155.77 (136.73-180.08) mg/cm^3, a total cortical BMD of 157.55 (144.8-166.94) mg/cm^2 and a total integral BMD of 309.29 (280.3-324.62) mg/cm^3. Cortical thickness (mm) was 1.89 (1.85-2.01). Table 2 includes densitometric data and 3D-Shaper parameters of patients with HPP.

Demographic, clinical, densitometric and 3D-Shaper data of subjects with PPH with fracture versus without fracture

In the first place, the comparison of demographic, clinical, densitometric and 3D-Shaper data of the five subjects with HPP who presented stress fractures compared to the 28

who did not. The patients with stress fractures were women, 40% postmenopausal, with a mean age of 46.35±10.1 years, while in the group of patients without them, 57.14% were women (25% postmenopausal) with a mean age of 51.31±15.82 years (p=0.48). Differences were observed in the prevalence of women, higher in the first group (p=0.07) and in BMI, lower in patients with stress fractures (23.5±2.44 kg/cm^2) compared to the other group (26.81±4.5 kg/cm^2; p=0.07). There were no differences in the rest of the risk factors for osteoporosis. Although no differences were observed in BMD parameters in FP, the 3D-Shaper analysis showed a statistically significant difference in cortical thickness (mm), which was less in those patients with stress fractures [1,8 (1 .77-1.89)] versus the non-fractured [1.94 (1.87-2.03); p=0.03)].

Second, the 9 patients with a history of traumatic fracture were compared to the 19 without a history of fracture, and no densitometric differences were observed or in 3D-Shaper parameters (p>0.05). Third, in the comparison between subjects with stress fractures and those who presented fractures of traumatic etiology, a decrease in cortical thickness (mm) was observed, lower in patients in the first group [1.8 (1.77-1.89)] versus the second [1.97 (1.88-2.03), p=0.03]. No differences were observed in the rest of the densitometric parameters or DXA-3D.

From a biochemical point of view, there were no significant differences in alkaline phosphatase, PTH and vitamin D levels between groups (p>0.05). Table 3 shows the intergroup comparison of demographic, clinical, densitometric and 3D-Shaper data, and figure 1 shows the levels of AF stratified by groups.

Discussion

In this work, a descriptive analysis of the densitometric characteristics of adults with HPP and of the bone architecture parameters was carried out using 3D-Shaper, a technique still little explored in these patients, as well as a comparison of these parameters between patients with and without a history of fractures.

Table 1. Demographic characteristics and risk factors for osteoporosis of patients with HPP

Characteristics	TG+ (N=33)
Age (years), median (IQR) Age (years), mean ± SD	51.01 (37.96-63.02) 50.56 ± 15.08
Female gender, n (%)	21 (63.6%)
Postmenopausal women, n (%)	9 (42.9%)
Men >50 years, n (%)	8 (66.6%)
BMI (kg/m^2), median (IQR) BMI (kg/m^2), mean ± SD	25.91 (22.89-29.25) 26.31 ± 4.39
Caucasian race, n (%)	33 (100%)
Calcium intake (g), median (IQR) Calcium intake (g), mean ± SD	400 (250-500) 401.52 ± 152.32
Smoking habit, n (%)	6 (18.2%)
Alcohol intake ≥30 g, n (%)	0%
Regular exercise, n (%)	19 (57.6%)
Solar exposure, n (%)	19 (57.6%)
Family history of hip fracture, n (%)	4 (12.1%)
PH traumatic fracture, n (%)	10 (30.3%)
PH fragility fracture, n (%)	0 (0%)
PH stress fractures, n (%)	5 (15.2%)
APH (IU/L), median (IQR) APH (IU/L), mean ± SD (RN: 46-116 IU/L)	25 (20.5-27.5) 25.2 ± 6.53
PTH (pg/ml), median (IQR) PTH (pg/ml), mean ± SD RN: 18.5-88 pg/ml	37 (30.5-64) 44.82 ± 22.41
Vit. D (ng/ml), median (IQR) Vit. D (ng/ml), mean ± SD (RN: 30-100 ng/ml)	19 (13-23.5) 20 ± 9.75

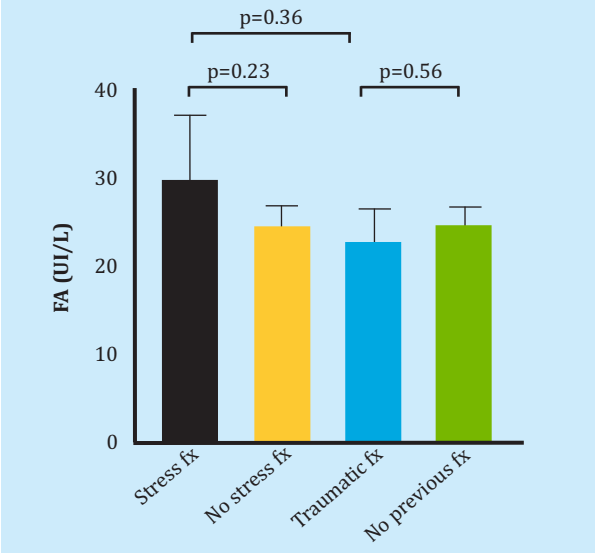
Quantitative data are expressed as median (interquartile range, IQR) and mean ± standard deviation (SD). The qualitative ones as absolute numbers and percentages. p<0.05 is considered significant. TG+: patients with persistent hypophosphatasemia and a positive genetic test confirming hypophosphatasia; BMI: body mass index; PH: personal history; APH: alkaline phosphatase; PTH: parathyroid hormone; Vit. D: vitamin D; NR: normal range.

Table 2. Densitometry and 3D-Shaper data in patients with HPP

Densitometric and 3D SHAPER data	TG+ (N=33)
BMD femoral neck (g/cm^2)	0.876 (0.83-0.92)
Z-score femoral neck	-0.29 (-1.13-0.25)
Diaphysis BMD (g/cm^2)	1.123 (1.03-1.21)
Trochanteric BMD (g/cm^2)	0.759 (0.693-0.82)
Trochanteric Z-score	-0.4 (-1.05-0.1)
BMD total hip (g/cm^2)	0.931 (0.89-1)
Total hip Z-score	-0.06 (-0.79-0.39)
Total cortical VBMD (mg/cm^3)	813.45 (759.12-862.26)
Total cortical SBMD (mg/cm^2)	157.55 (144.80-166.94)
Total trabecular VBMD (mg/cm^3)	155.77 (136.73-180.08)
Total integral VBMD (mg/cm^3)	309.29 (280.3-324.62)
Cortical thickness (mm)	1.89 (1.85-2.01)

Data are expressed as median (interquartile range, IQR). TG+: patients with persistent hypophosphatasemia and a positive genetic test confirming hypophosphatasia; BMD: bone mineral density; VBMD: volumetric bone mineral density; SBMD: superficial bone mineral density; p<0.05 is considered statistically significant.

Figure 1. Alkaline phosphatase levels stratified by groups according to previous fracture history



Alkaline phosphatase levels expressed as median (interquartile range) stratified by groups. FA: alkaline phosphatase; Fx: fracture.

In our study we found that densitometric alterations were not particularly relevant in terms of the prevalence of osteoporosis. The repercussion has resulted in more moderate changes in these patients, with a high prevalence of osteopenia, more pronounced in postmenopausal women and men over 50 years of age. The slight decrease in BMD observed in this study seems to be in accordance with the results found in the existing literature on adults with HPP, in which the majority of patients presented normal parameters or a slight decrease in Z-score values, findings that seem respond to milder forms of the adult[5,7,9].

Previous studies have not found differences in BMD in subjects diagnosed with HPP with and without fractures, suggesting that this test may not adequately translate the risk of presenting them[7]. Nor in our study have we found significant differences in the densitometric analysis between individuals with HPP with and without a history of stress fractures. However, the 3D-Shaper technique shows a statistically significant decrease in cortical thickness (mm) at the FP level in patients with stress fractures [1.8 (1.77-1.89)] compared to those without this history. [1.94 (1.87-2.03, p=0.03)] and compared to those with traumatic fractures [1.97 (1.88-2.04), p=0.03] that does not seem to be explained by a lower level of alkaline phosphatase.

In this same disease, applying high-resolution peripheral quantitative computed tomography (HR-pQCT) in the left distal tibia and right distal radius, Schmidt et al. also reported a decrease in cortical thickness in patients with HPP with fractures compared to those without fractures[7]. Likewise, other work has highlighted the presence of a decrease in cortical thickness in the radiographs of some adult patients with HPP[18].

Although we have not found evidence from other studies that analyze 3D-Shaper parameters in HPP, other publications have evaluated this technology in patients with different bone metabolic pathologies. Gracia-Marco et al.[19] observed differences in cortical thickness in subjects with primary hyperparathyroidism, lower in patients with this disease compared to healthy controls (1.85±0.14 mm vs. 1.93±0.17 mm ; p=0.023). These results suggest that bone architecture studies could be of special interest with other diseases involving high bone remodeling. However, Humbert et al.[20] observed a non-significant decrease in cortical thickness in postmenopausal patients with hip fracture compared to controls (1.746±0.127 mm vs. 1.783±0.123 mm; p=0.1). In our study, patients with HPP and traumatic fracture did not show a decrease in cortical thickness compared to those who did not fracture.

Stress fractures were originally described in military recruits and were considered "fatigue fractures" as a consequence of repeated and prolonged minimal or small mechanical impacts on a bone with normal elastic resistance. A subtype of stress fractures is insufficiency fractures produced by a normal load on a bone with altered resistance, described in patients with vitamin D deficiency (Looser-Milkman lines, characteristic of osteomalacia)[21,22]. A high prevalence of insufficiency fractures or pseudofractures has also been described in patients with HPP[1], but we do not know exactly which patients will develop them. Regarding their location, recurrent metatarsal fractures and femoral fractures and pseudofractures are characteristic, which are those found in our patients.

With probable multifactorial pathogenesis, stress fractures could reflect alterations in BMD and bone quality[23]. In our study, we did not find differences in BMD at the PF level of patients who had stress fractures versus those who did not, coinciding with what was recently published by other authors[11]. The decrease in cortical thickness observed in our patients with stress fractures would reinforce the existence of a qualitative bone alteration. López Delgado et al.[12] describe low bone remodeling in patients with persistent hypophosphatasemia, although this does not seem to translate into differences in BMD or trabecular bone score (TBS) when compared with a control group. Our patients with stress fractures did not show differences in the level of alkaline phosphatase decrease compared to those who do not fracture, so it is difficult to explain the presence of fractures due to a greater severity of the enzyme defect.

As limitations of our study, most of our subjects present heterozygous mutations that condition milder forms of the disease. From the densitometric point of view, that, up to now, we do not have the reference population values for 3D-Shaper measurements, they have not been compared with a control group, a fact that may limit how the results are interpreted. As strengths, however, it is worth highlighting the significant number of patients, given that this is a rare disease and studied using a new technique.

These data seem to reflect a discrete impact at the densitometric level in the mildest adult forms. A decrease in cortical thickness was identified in patients with HPP with stress fractures. Bone architecture studies in PF could be of interest to determine subjects with HPP susceptible to presenting this type of fracture.

Ethics Committee approval: All the studies carried out followed the principles set forth in the Helsinki declaration and were formally approved by the La Paz Hospital Clinical Trials Committee (PI 3239). Informed consent has been obtained from all patients.

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Table 3. Demographic, clinical, densitometric and 3D-Shaper data of subjects with versus without fracture

	1 With fractures of stress (n=5)	2 No fractures of stress (n=28)	3 With fracture traumatic (n=9)	4 No fracture (n=19)	P value 1-2	P value 3-4	P value 1-3
Age (years), median (IQR) Age (years), mean ± SD	43.76 (37.56-56.46) 46.35 ± 10.1	52.78 (37.6-64.25) 51.31 ± 15.82	56.58 (38-63.03) 51.12 ± 12.21	51,01 (36,68-69) 51,01 ± 17,58	0.48	0.96	0.36
Female gender, n (%)	5 (100%)	16 (57.14%)	4 (44,4%)	12 (63,2%)	0.07	0.35	0.04
Postmenopausal women, n (%)	2 (40%)	7 (25%)	1 (25%)	6 (66,7%)	0.88	0.38	0.63
Men >50 years, n (%)	0	10 (100%)	4 (80%)	4 (50%)	0.51	0.41	-
BMI (kg/m^2), median (IQR) BMI (kg/m^2), mean ± SD	22.6 (21.81-25.67) 23.5 ± 2.44	26.33 (23.19-29.9) 26.81 ± 4.5	26.22 (23.44-29.8) 26.22 ± 3.56	26.4 (23.14-31.2) 27 ± 4.96	0.07	0.89	0.11
Calcium intake (g), median (IQR) Calcium intake (g), mean ± SD	400 (250-550) 400 ± 158.11	400 (225-500) 401.8 ± 154.25	300 (200-500) 350 ± 150	450 (300-500) 450 ± 153.99	1.00	0.29	0.61
Smoking habit, n (%)	2 (40%)	4 (14.29%)	0%	4 (66.7%)	0.17	0.13	0.04
Alcohol intake, n (%)	0%	0%	0%	2 (33.3%)	0.91	-	0.59
Regular exercise, n (%)	3 (60%)	16 (57.14%)	7 (77.8%)	9 (47.4%)	0.91	0.13	0.48
Solar exposure, n (%)	3 (60%)	16 (57.14%)	5 (55.6%)	11 (57.9%)	0.91	0.91	0.87
FH hip fracture, n (%)	0%	4 (14.29%)	1 (11.1%)	3 (75%)	0.37	0.74	0.44
APH (UI/L), mediana (RIQ) APH (UI/L), media ± DE (RN: 46-116 UI/L)	30 (21-37) 29.2 ± 9.45	25 (20.25-27) 24.36 ± 5.1	23 (20.5-26.5) 24.22 ± 7.1	25 (20-27) 24.42 ± 4.06	0.23	0.56	0.36
PTH (IU/L), median (IQR) PTH (IU/L), mean ± SD (RN: 46-116 IU/L)	29 (19.5-43.5) 31 ± 12.9	38 (31.5-64) 47.29 ± 23	40 (36.5-65) 45.44 ± 16.88	37 (31-64) 48.16 ± 25.77	0.1	0.56	0.15
Vit. D (ng/ml), median (IQR) Vit. D (ng/ml), mean ± SD (RN: 30-100 ng/ml)	30 (14-42.5) 28.6 ± 17.2	18.57 (13-22.75) 18.57 ± 7.31	13 (11.5-21.5) 17.67 ± 9.87	19 (16-23) 19 ± 6	0.14	0.24	0.24
BMD neck femur (g/cm^2)	0.88 (0.75-0.91)	0.88 (0.83-0.94)	0.91 (0.82-1.04)	0.87 (0.83-0.9)	0.48	0.59	0.3
Z-score neck femur	-0.56 [-1.5-(-0.17)]	-0.12 (-1.12-0.3)	-0.1 (-1.18-0.36)	-0.29 (-1-0.32)	0.34	0.92	0.36
Diaphysis BMD (g/cm^2)	1.02 (1.005-1.17)	1.13 (1.06-1.2)	1.18 (1.07-1.24)	1.13 (1.04-1.21)	0.29	0.47	0.24
Trochanteric BMD (g/cm^2)	0.74 (0.68-0.8)	0.78 (0.69-0.82)	0.79 (0.73-0.85)	0.74 (0.68-0.8)	0.55	0.25	0.19
Trochanteric Z-score	-0.5 (-0.85-0.2)	-0,3 (-1,08-0,2)	-0.6 (-1.05-0.15)	-0.2 (-1.1-0.2)	0.9	0.96	0.7
Total BMD (g/cm^2)	0.88 (0.85-0.99)	0.95 (0.9-1.01)	0.99 (0.91-1.04)	0.93 (0.89-1)	0.29	0.44	0.24
Total Z-score	-0.06 (-0.78-0.3)	-0.07 (-0.8-0.4)	-0.1 (-0.75-0.22)	-0.05 (-0.86-0.65)	0.94	0.85	1
Cortic VBMD (mg/cm^3)	844.64 (762.14-879.22)	804.3 (758.06-860.12)	795.14 (768.14-864.74)	813.45 (757-859.97)	0.48	0.6	0.8
Total cortical SBMD (mg/cm^2)	155,61 (136,77-162,5)	156.97 (146.18-168.44)	164.01 (148.97-169.07)	157.54 (142.29-166.77)	0.42	0.33	0.24
Total trabecular VBMD (mg/cm^3)	154.43 (122.66-189.92)	156.86 (139.41-178.91)	164.71 (136.03-191.63)	155.77 (142.25-178.04)	0.84	0.84	0.7
Total integral VBMD (mg/cm^3)	297.28 (267.83-352.33)	309.77 (279.28-322.21)	306.32 (282.58-341.22)	310.25 (277.73-323.31)	0.92	1	0.8
Cortical thickness (mm)	1.8 (1.77-1.89)	1.94 (1.87-2.03)	1.97 (1.88-2.04)	1.88 (1.86-2.03)	0.03	0.44	0.03

Quantitative data are expressed as mean and standard deviation (SD) and median, interquartile range (IQR) and qualitative data as frequencies and percentages. BMI: body mass index; FH: family history; APH: alkaline phosphatase; PTH: parathyroid hormone; vit. D: vitamin D; BMD: bone mineral density; VBMD: volumetric bone mineral density; SBMD: superficial bone mineral density; $p<0.05$ is considered statistically significant.

Conflict of interest: This study has been funded by an unrestricted grant from Alexion Pharmaceuticals Inc. for the study of hypophosphatasia of the adult whose main investigator is Dr. P. Aguado and sub-investigator Dr. C. Tornero and the application of the 3D-Shaper Software has been carried out within the framework of a multicenter study (SEIOMM-3D-DXA) developed by the Spanish Society for Bone Research and Mineral Metabolism (SEIOMM). L. Humbert is a shareholder and employee of the Galgo Medical company. C. Tornero, M. Coronado, V. Navarro-Compán, S. García, C. Lancha, A. Balsa and P. Aguado declare that they have no other conflicts of interest.

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Cx43 and primary cilium involvement in osteocyte activity

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Summary

Objetives: Bone tissue can adapt to environmental stimuli by altering its morphology and metabolism. Different bone cells communicate with each other through communicating junctions (CJs). Connexin 43 (Cx43) is the most abundant CJ protein with key functions in signal transduction and in response to hormonal and mechanical stimuli. Another mechanosensor element of osteocytes is the primary cilium, formed by microtubules and which develops in the cell cycle's G0 phase. Our study aims to determine Cx43 and primary cilium involvement in osteocytic activity, to analyze the possible interaction between these two mechanosensors and to assess the role they play in the detection and response of osteocytes to mechanical stimuli and stimulation of the parathormone type 1 receptor (PTH1R) by its ligand, the parathormone-related protein (PTHrP) (1-36).

Material and methods: The control MLO-Y4 (Cx43 +/+) osteocyte cell line was compared to Cx43-deficient MLO-Y4 (Cx43 -/-). The expression analysis of intraflagellar transport protein 88 (IFT88), Cx43 and phosphorylation of the extracellular signal regulatory kinase (P-ERK) was determined by Western blot. To characterize the possible colocalization between the primary cilium and Cx43, an immunofluorescence was carried out. To simulate mechanical stimulation in vitro, cells were subjected to mechanical stress of 10 dynes/cm2 by fluid flow for 10 minutes.

Results: The results obtained show that the number of cells with primary cilium does not vary due to the expression of Cx43 ($p = 0.089$). In cells with Cx43 presence, mechanical stimulation by fluid flow and PTHrP increase the phosphorylation of extracellular signal-regulated kinases (ERK) compared to unstimulated cells ($p = 0.049$ and $p = 0.011$, respectively).

Conclusions: The primary cilium and Cx43 act as mechanosensing elements for osteocytes. Deficiency in Cx43 does not influence ciliogenesis or activation by mechanical stimulation of pro-survival signaling pathways in osteocytes.

Key words: osteocytes, connexin 43, primary cilium, mechanical stimulus, PTHrP.

Introduction

Bone tissue has the ability to adapt to surrounding environmental stimuli by altering its morphology and metabolism[1].

The development, remodelling and repair of this tissue are dynamic processes, regulated by the joint activity of bone cells (osteocytes, osteoblasts and osteoclasts). Osteocytes are the most abundant type of cells in the bone. They are located in the mineralized bone matrix, forming a large cellular intercommunication network, called osteocyte lacuno-canalicular system (OLCS). Osteocytes are the main mechanosensory cells in the bone[2]. They can detect mechanical stimuli in the environment and communicate this signal to effector cells (osteoblasts and osteoclasts) and have different mechanosensory structures: ion channels, integrins[3], parathyroid hormone receptor type 1 (PTH1R) ligands, connexins[4] and primary cilia. Some of these mechanosensors have been found to interact with each other, allowing the integration of multiple extracellular signals[3].

Mechanical stimuli regulate bone remodelling. The deregulation of this process produces osteoporosis, a condition characterized by decreased bone mass and increased bone frailty[5].

Osteocytes respond to mechanical stimulation by activating different signalling pathways, such as Wingless-type protein (Wnt)HPPβ-catenin, and mitogen-activated protein kinases (MAPK) and Hedghog (HH). In this work, we analized some of the molecules acting in these signalling pathways, specifically P-ERK and ERK proteins. Interaction among the cells constituting the bone tissue is essential for bone tissue homeostasis[6].

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Cell communication via gap junctions (GJs) is one of the most important of these interactions, as it happens between cytoplasms of adjacent cells and, therefore, allows intercellular diffusion of small molecules[7]. GJs not only serve as passive channels, they also intervene in the regulation of different signaling routes[8].

Cxs are transmembrane proteins, named after their molecular weight, from 26 to 59 kDa. Cx43 is the most abundant protein in the GJs of bone cells. Connexins, in particular Cx43, interact with structural and signalling molecules, regulating cellular functions[9,10].

Primary cilia are microtubule-based structures in which numerous receptor channels and proteins are located, which allow the cilia to act as a mechanosensors[11,12]. PTH1R is a G-protein-coupled receptor (GPCR), which is expressed in primary cilia and plays a fundamental role in mechanical signal transduction in MLO-Y4[13] cells. This receptor has two widely characterized ligands: PTH and PTHrP (parathormone-related protein). Both PTH and PTHrP have effects on bone formation and are used as anabolic agents in the treatment of osteoporosis[13,14].

It has also been shown that stimulation by PTHrP and mechanical stimulation by fluid flow induce the activation of ERK, thus preventing the increase of osteocyte apoptosis[15].

In the present study, it was hypothesized that primary cilia and Cx43 act jointly in the regulation of signalling pathways involved in cell survival and in cell adhesion capacity. The expression of primary cilia was determined both in Cx43 +HPP+ and Cx43 -HPP- cells and the non-colocalization of these two mechanosensors. Therefore, it is suggested that Cx43 deficiency is not involved in the development of the primary cilia, but could influence other aspects, such as their functionality, length or intra-flagellar transport.

Likewise, the cellular response of osteocytes (phosphorylation of ERK) was analyzed after stimulating PTH1R, both with PTHrP, obtaining a greater increase in P-ERK in Cx43 -HPP- cells as opposed to in Cx43 +HPP+, and mechanically, which produced an increased P-ERK expression regardless of Cx43 deficiency.

Materials and methods

1. Cell cultures

In this project, we worked with the continuous line of murine long bone MLO-Y4 osteocytes Cx43 +HPP+, as a control, and deficient in Cx43 (Cx43 -HPP-), which were kindly provided by Dr. L. I Plotkin.

The cells were cultured at a concentration of 24,000 cellsHPPcm^2, with α-Modified Eagle's Medium (α-MEM) (Gibco™, Thermo Fisher Scientfic, Spain), supplemented with 2.5% calf serum (Calf Serum; CS), 2.5% fetal bovine serum (FBS), 1% L-Glutamine, 1% PenicillinHPPStreptomycin and Puromycin (from Streptomyces alboniger, Sigma Aldrich, BioReagent, Merck, Spain) at a concentration of 10 µgHPPml.

To promote the development of the primary cilium, the cells were grown in depletion medium, composed of α-MEM (Gibco™) supplemented with 1% penicillinHPPstreptomycin and puromycin at a concentration of 10 µgHPPml, for 24 hours.

All surfaces on which these cells were cultured had to be previously collagenized, using for this matter type I collagen at 0.01% acetic acid, to simulate the collagen matrix where the osteocytes are embedded in vivo. Cells were kept at 37ºC and 5% CO_2.

2. Western blot

The cellular total protein was extracted using RIPA buffer (Sigma-Aldrich, Merck, Spain), supplemented with protease and phosphatase inhibitors (Calbiochem, Merck, Spain).

Subsequently, these proteins were quantified using bicinchoninic acid (BCA) (PierceTM BCA Protein Assay Kit, Thermo Fisher Scientfic, Spain), which generates a colorimetric reaction detectable at 562 nanometres (nm). To carry out the reading, the Varioskan Flash plate reader (Thermo Fisher Scientfic) was used, with SkanIt Software 2.4.3 RE.

Protein extracts (20 µg) were separated by means of a 10% polyacrylamide gel under reduced conditions. They were later transferred to a nitrocellulose membrane (Bio-Rad, Hercules, California, USA). The blocking was carried out with 5% milk powder, in Tris-saline buffer with 0.05% Tween20 (TTBS), for 1 hour under stirring at room temperature. The following primary antibodies were then stirred between 15-18 hours at 4ºC: anti-Phospho-p44HPP42 MAPK (Erk1HPP2) (Cell Signaling, Beverly, Massachusetts, USA), anti-p44HPP42 MAPK (Erk1HPP2), anti-Cx43 (Sigma Aldrich, ST. Louis, Missouri, USA) and anti-tubulin (Sigma Aldrich). These are all polyclonal antibodies produced in rabbits, except for anti-tubulin which is a monoclonal antibody produced in mice. It was then incubated for one hour at room temperature, with the corresponding IgG coupled to peroxidase, and the membrane was developed by chemiluminescence with Clarity™ Western ECL substrate (Bio-Rad, Life Science Research, Spain). The intensity of the bands was quantified by densitometry, using the DNR Bio Imaging System MF ChemiBIS3.2 and Gelcapture and QuantityOne™ (Bio-Rad) programs.

3. Immunofluorescence

30,000 cellsHPPwell were cultured from the multiwell plates (Falcon®, Thermo Fisher Scientfic, Spain). The cells were grown until they reached 80% confluence; and subsequently depletion medium was added for 24 hours to induce the formation of the primary cilia. The cells were then fixed with 4% paraformaldehyde (PFA) and permeabilized with Triton X-100 at 0.5%. Next, the blocking solution, composed of bovine serum albumin (BSA) at 10%, supplemented with goat serum at 5%, was added for 1 hour. After that, the following primary antibodies were stirred for 15-18 hours at 4ºC: polyclonal anti-Cx43 produced in rabbits (Sigma Aldrich) and monoclonal anti-α acetylated tubulin produced in mice (Sigma Aldrich), to observe the primary cilium. The secondary antibodies were then arranged: Alexa fluor® 488 goat anti-mouse cilium (Invitrogen Molecular probes, Thermo Fisher ScientificTM, Spain), and Cx43, Alexa fluor® 568 anti-rabbit IgG (Life technologies, Thermo Scientific™, Spain) (1: 1000 dilution in BSA at 10% and goat serum at 5%). After 1 h of incubation, 4'-6-diamidino2-phenylindole (DAPI) was added. Nuclei, the primary cilia and Cx43 were seen on the fluorescence microscope (Leica CTR 6000). The merging of the individual images of the primary cilium, Cx43 and cell nuclei into one single image was performed using the ImageJ program.

4. Mechanical stimulation by fluid flow (FF) and by PTHrP

To perform stimulation by FF and PTHrP, cells were cultured on teflon-bound glass slides at a density of 25,000

cellsHPPcm^2. The FF technique is based on the constant bombing of culture medium over the cell monolayer using a peristaltic pump (Flexcell International Corp., Hillsborough, North Carolina, USA.) at 10 dynesHPPcm^2 in a hermetically closed circuit for 10 minutes. The time and frequency settings were established using the Master Flex Peristaltic Pump 2010 program. On the other hand, stimulation was performed with PTHrP (1-36) (Bachem, Bubendorf, Switzerland) at a concentration of 10^{-7} molar (M), for 10 min. The same number of cultures, but with cells that were not subjected to any stimuli, served as static controls (SC).

5. Statistical analysis

In the statistical analysis of the results, data are expressed as mean ± standard deviation of at least two experiments carried out in triplicate. It was performed using the GraphPad Prism 8 program (GraphPad software, La Jolla, California, USA). To compare means between two groups, the nonparametric Mann-Whitney test was carried out, and to compare means of more than two groups, the non-parametric Kruskal-Wallis test was used. For mulptiple pairwise comparison we used Dunn's test. The established confidence interval was 95% in all statistical tests. So the results with a value of $p<0.05$ were considered statistically significant.

Results

1. Effect of Cx43 on the development of the primary cilium in Cx43 +HPP+ and Cx43 -HPP- cells

The results obtained by Western blot indicate that in Cx43-deficient cells the expression of this protein decreases significantly in comparison with control cells Cx43 +HPP+ (W=0, p=0.029), which allows us to confirm the deficiency of this protein (Figure 1). In addition, the presence of the primary cilium was characterized by the expression analysis of the IFT88 protein. IFT88 was used as a marker for the primary cilium presence, since it is a highly abundant protein in this organelle, as it is involved in intraflagellar transport, needed for ciliogenesis[18]. We observed that the IFT88 protein expresses in a similar way, regardless of the deficiency in Cx43 (W=4, p=0.343).

Likewise, Cx43 and primary cilia were analyzed by immunofluorescence to try to determine the possible interaction between these two mechanosensors. Figure 2 shows both Cx43 +HPP+ and Cx43 -HPP- cells develop a primary cilium, which is evidenced by the presence of the primary anti-α-acetylated tubulin antibody, which originates from the cell surface. Also, it can be seen that Cx43 appears mainly in the cell membrane, possibly forming GJs. Furthermore, it appears that the primary cilium does not co-localize with Cx43. On the other hand, we performed counts of the cells with primary cilium from nine photographs taken in different fields using the fluorescence microscope. In each image the number (No.) of total cells (cel.) and the number of cells with a presence of primary cilium were quantified, and we calculated the ratio (No. of cells with ciliumHPPNo. of total cells), both for the Cx43 +HPP+ and for Cx43 -HPP- cell lines (Table 1 and Figure 3). In these results, we found that the number of cilia formed did not significantly differ (p=0.089) between the two cell lines (Cx43 +HPP+ and Cx43 -HPP-).

2. Effect of mechanical stimulation and PTHrP on Cx43 +HPP+ and Cx43 -HPP- cells

In several studies it has been determined that mechanical stimulation inhibits osteocyte apoptosis, through a mechanism that involves phosphorylation of MAPKs as ERKs[16]. The aim of this experiment was to analyze whether Cx43 deficiency altered the effect of mechanical stimulation on osteocytes, and to determine whether Cx43 is involved in the activation of the PTH1R receptor, after stimulated by one of its PTHrP ligands. The effects triggering mechanical stimulation and PTHrP stimulation in MLO-Y4 cells, the P-ERK expression was analyzed using Western blot.

Figure 1. Expression of Cx43 and IFT88. A) Result of the nitrocellulose membrane development for the analysis of Cx43, IFT88 and tubulin as loading control; total cellular protein extracts were used (25 µg). The molecular weight (MW) marker was placed in the first lane; the next four lanes belong to four replicas of control MLO-Y4 cells (Cx43 +HPP+) and the last four correspond to four replicas of Cx43-deficient MLO-Y4 cells (Cx43 -HPP-). B) Mean ± standard deviation of Cx43HPPtubulin. C) Mean ± standard deviation of IFT88HPPtubulin. Protein levels normalized in contrast to tubulin *p<0.05

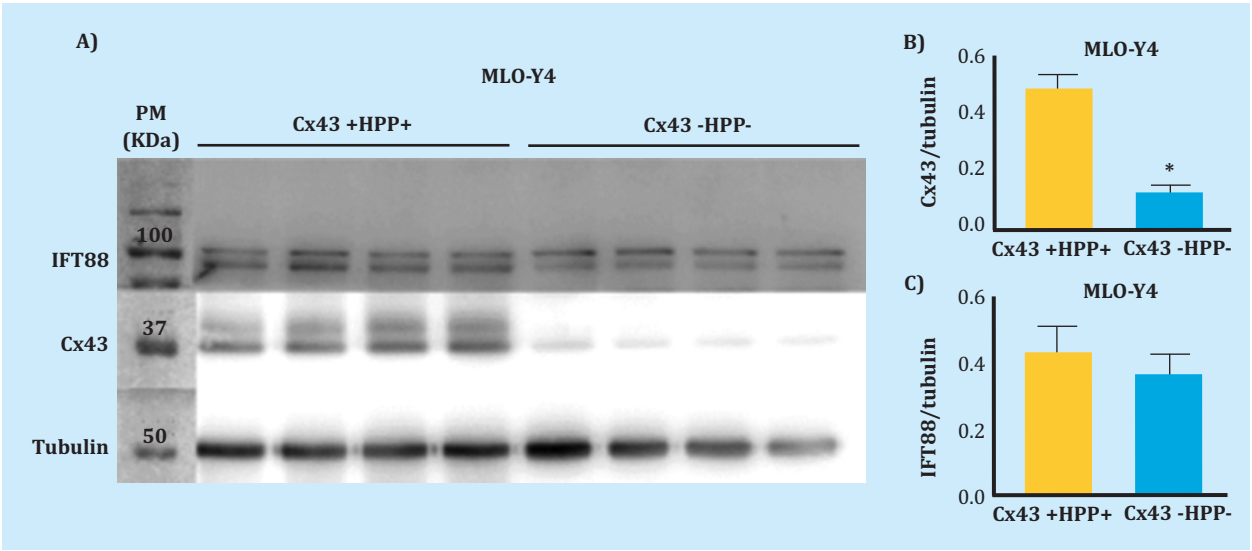


Figure 2. Immunofluorescence performed with osteocytes of the MLO-Y4. A) Cx43 +HPP+ and B) Cx43 -HPP- line. Cell nuclei were stained with DAPI (blue), anti α-acetyl-tubulin antibodies were used to mark the primary cilium (green), anti-Cx43 antibody to mark Cx43 (red) and merged. The images were taken with an immersion objective at 63X. Scale bar = 16 µm

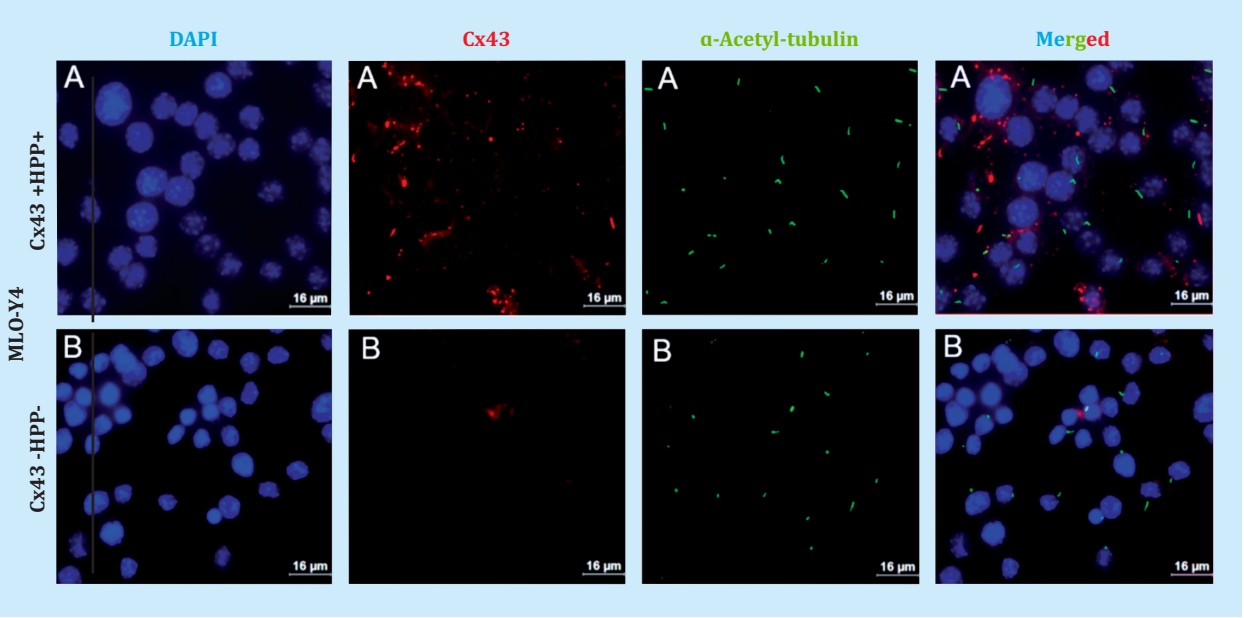


Table 1. Counts of cells (cel.) with presence of primary cilium in the MLO-Y4 Cx43 +HPP+ and Cx43 lines

No. of photo	Cel. number totals Cx43 +HPP+	Cel. number with cilium Cx43 +HPP+	Cel. number with cilium/cell number totals Cx43 +HPP+ (%)	Cel. number totals Cx43 -HPP-	Cel. number with cilium Cx43 -HPP-	Cel. number with cilium/cel. number Cx43 -HPP- (%)
1	96	43	44.792	94	41	43.617
2	93	44	47.312	84	36	42.857
3	95	41	43.158	93	20	21.505
4	110	51	46.364	77	33	42.857
5	103	56	54.369	91	24	26.374
6	77	32	41.558	125	55	44.000
7	66	33	50.000	106	61	57.547
8	95	44	46.316	104	49	47.115
9	111	52	46.847	195	69	35.385

Figure 3. No. of cells with ciliumHPPNo. of total cells (%) in the Cx43 +HPP+ and Cx43 -HPP- lines. Results are expressed as mean ± standard deviation

	Cx43 +HPP+	Cx43 -HPP-
Half	46.746	40.139
Typical deviation	3.757	10.913
Standard error	0.129	0.351

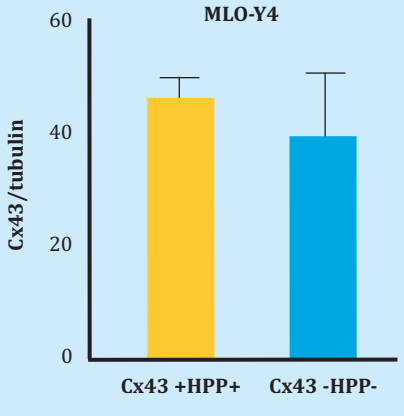
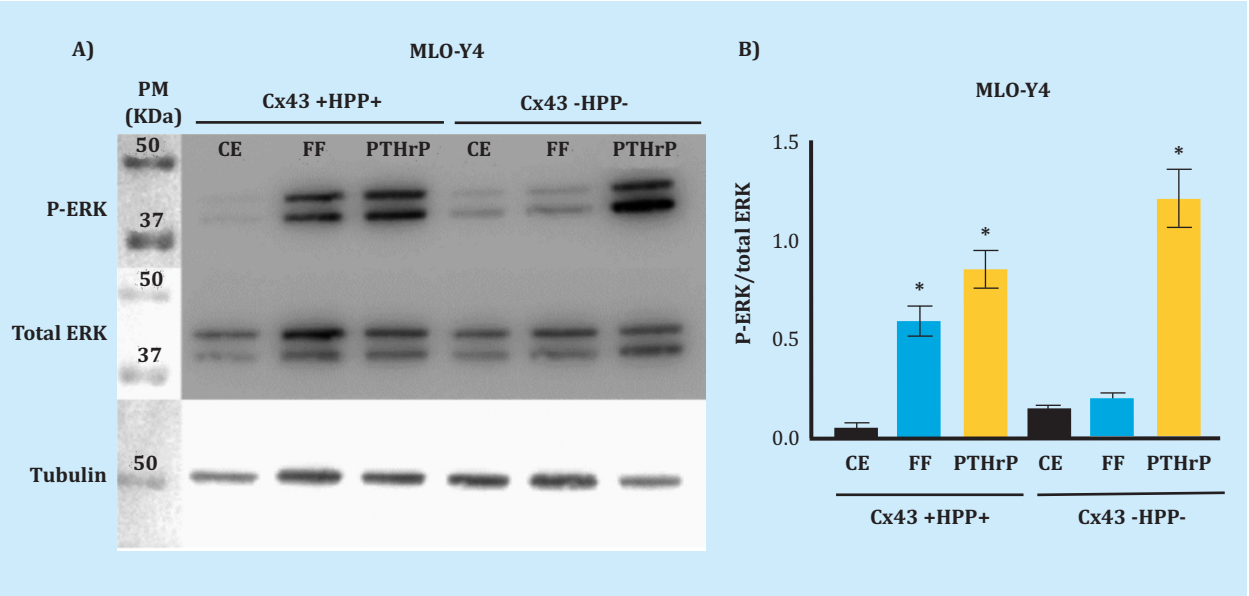


Figure 4. P-ERK analysis after mechanical stimulation and with PTHrP. After mechanical stimulation and stimulation with PTHrP, changes in protein levels were evaluated by Western blot, and those regarding P-ERK in total cell protein extracts, cell lines Cx43 +HPP+ and Cx43 -HPP-, stimulated or not (static control, SC). Total ERK and tubulin were used in order to normalize. Results are expressed as mean ± standard deviation of a duplicate experiment of each condition vs. SC



The Kruskal-Wallis test was applied to establish the differences between the Cx43 +HPP+ and Cx43 -HPP- groups for the conditions: SC, FF and PTHrP. The test result pointed at differences between some of the groups (p=0.0005). To determine between which groups, Dunn's Multiple Comparison Test was performed, showing that both mechanical stimulation (FF) (p=0.049) and PTHrP stimulation (p=0.017) induce a significant increase in ERK phosphorylation in contrast to non-stimulated cells (static control, SC); in the MLO-Y4 Cx43 +HPP+ cell line. This result shows that extracellular stimuli favour the activation of the SrcHPPERK signaling pathway, which promotes cell survival. In the case of MLO-Y4 Cx43 -HPP- we observed that P-ERK does not increase significantly in contrast to CE (p=0.955) after mechanical stimulation (FF). However, when stimulated with PTHrP, ERK phosphorylation increases significantly (p=0.025) (Figure 4).

Discussion

Connexins and primary cilia are bone cells' mechanosensory elements which play a fundamental role in the stimuli detection and signal transmission[3].

The results obtained by Western blot confirm that the Cx43 -HPP- cell line used in the experiment was deficient in this protein, since the level of Cx43 expression decreased significantly compared to that on the Cx43 +HPP+ line. The IFT88 protein expression was also analyzed, since it was used as a marker for the presence of the primary cilia in previous studies[17]. According to the results obtained, it can be concluded that there are no significant differences in the IFT88 protein expression between the Cx43 +HPP+ and Cx43 -HPP- lines. The results of the IFT88 analysis by Western blot alone do not allow us to ensure that primary cilia develops correctly, because the IFT88 protein could be expressing itself in another cell compartment different from the primary cilia. Therefore, to verify whether the deficiency in Cx43 influences the formation of the primary cilia, the expression of this organelle and of Cx43 was analyzed by immunofluorescence. Both Cx43 +HPP+ cells and Cx43 -HPP- cells were observed to develop primary cilia; Likewise, it was found that the number of cilia formed did not differ meaningfully between the two cell lines. Furthermore, it seems to be observed that the Cx43 and the primary cilium do not co-localize; and in Cx43 +HPP+ cells, Cx43 is found in the plasma membrane of the cell, which would be the expected location, since it is where it forms GJs.

Previous research has shown that MLO-Y4 cells are an optimal model for mechanical stimulation studies[16]. However, in order to extrapolate the results obtained in these in vitro studies to the authentic in vivo conditions, it is necessary to work with mechanical stimuli that reproduce and generate responses similar to those that occur in the physiological situation. Currently, fluid flow (FF) over a monolayer of osteocytic cells is the technique that most closely approaches this situation[18].

The molecular target chosen as an indicator of the viability of the MLO-Y4 line was P-ERK, because its activation after mechanical stimulation is an indicator of survival in osteocytic cells[16,18]. In accordance with these investigations, mechanical stimulation by FF was found to induce an increase in the expression of P-ERK[16,18]. Thus, FF promotes the survival of MLO-Y4 cells.

On the other hand, the PTHrPHPPPTH1R system is also essential to regulate bone remodelling. Preliminary results suggest that PTH1R is key in the anabolic bone response consequent to mechanical stimulation in vivo. This receptor acts as a mechanoreceptor in osteoblast cells[13]. The results of the study carried out indicate that the exogenous administration of PTHrP (1-36) (PTH1R ligand) protects against apoptosis in a similar way to that of mechanical stimulation in osteocytes, since it induces an increase in the expression of P- ERK. Similarly, previous studies found that PTHrP, like PTH, has anti-apoptotic properties in osteocytes[16].

Conclusions

1. The number of cells with primary cilium does not vary due to the expression of Cx43.

2. The primary cilium and the Cx43 act as osteocytes mechanosensors.

3. The fluid flow-induced mechanical stimulation promotes the survival of MLO-Y4 cells, regardless of the deficiency in Cx43, since it causes an increase in the P-ERK expression, both in Cx43 +HPP+ and Cx43 -HPP- cells.

4. Exogenous administration of PTHrP (1-36) (PTH1R ligand) produces an increase in P-ERK in Cx43 +HPP+ and Cx43 -HPP- cells. However, this increase is much higher in Cx43 -HPP- cells. Therefore, we suggest Cx43 is inhibiting the PTH1R receptor, which, after binding its PTHrP ligand, causes the activation pathway in which P-ERK is involved not to fully trigger.

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

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Can a genetic condition be diagnosed based on phenotypic characteristics? A case of pseudohypoparathyroidism in Ecuador

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Summary
Pseudohypoparathyroidism is a rare disease of the endocrine gland. Its diagnosis should not be dismissed when hypocalcemia is accompanied by hyperphosphatemia and high levels of parathyroid hormone even if kidney failure and vitamin D deficiency do not occur. Although genetic studies provide a definitive diagnosis, biochemical tests that show hormonal resistance and phenotypic characteristics allow us to establish a diagnosis. Literature is limited in Latin America and few cases have been described. Here we report an 18-year-old male suffering pseudohypoparathyroidism and we discuss clinical characteristics, biochemical and radiographic findings, as well as treatment.

Key words: Albright's hereditary osteodystrophy, parathyroid hormone resistance, pseudohypoparathyroidism, inactivating PTH/PTHrP signaling disorder, hypocalcemia, brachydactyly, Ecuador.

Introduction

Pseudohypoparathyroidism (PHP) is a heterogeneous group of disorders which share in common a parathyroid hormone resistance (PTH).

Globally, estimated prevalence is 0.79/100,000[1], though it depends on the analysed type of PHP, and it oscillates between 6.7 and 3.3 cases per million inhabitants in Italy[2] and Japan[3] respectively. Between 2000 and 2019, 325 cases[4] have been described in worldwide literature, most of them in developed countries, in which in addition, PHP subtypes have been documented throughout genetic studies. 1a subtype is the most common, representing 70% of the cases[1]. 47 cases have been reported in Latin America between 2000 and 2020[5-10], the most frequent subtype being 1b, followed by 1a and 1c. Due to a lack of genetic studies, in some cases is not possible to precisely ascertain the belonging to one or another subtype[10].

The following is a description of an 18-year-old male's clinical case presenting phenotypical features of Albright's hereditary osteodystrophy (AHO).

Case report

An 18-year-old man attended an endocrinology consultation after being referred by the neurology service due to seizures associated with persistent hypocalcaemia. He presented a history of generalized tonic-clonic seizures from 15 years of age, for which he had been previously hospitalized, showing hypocalcaemia, treated with intravenous calcium and anticonvulsants to control the emergency and oral calcium supplements upon discharge from hospital. No further studies were carried out to determine the cause of hypocalcaemia.

His parents and first-degree relatives have no significant medical history. The patient is the child of a non-consanguineous marriage. He was born pre-term due to premature rupture of membranes at 35 weeks of gestation, with neonatal hypoxia and hypotonia. He presented late motor and language development, requiring language therapy from 5 to 7 years of age and physiotherapy since he was 2, in addition to school learning tardiness and permanent overweight. At 12 years of age he was diagnosed with primary hypothyroidism and since then he has taken 150 µg of levothyroxine sodium.

The patient is the youngest of 3 siblings who have no significant medical history.

On physical examination, he presented a characteristic phenotype: obese, short height, round face, prominent forehead, low nasal bridge, short neck, brachydactyly, and incomplete teeth (Figure 1). His weight was 68.8 kg, and his height, 153 cm (<3rd percentile); the body mass index was 34 (>97th percentile). His hands and feet were small,

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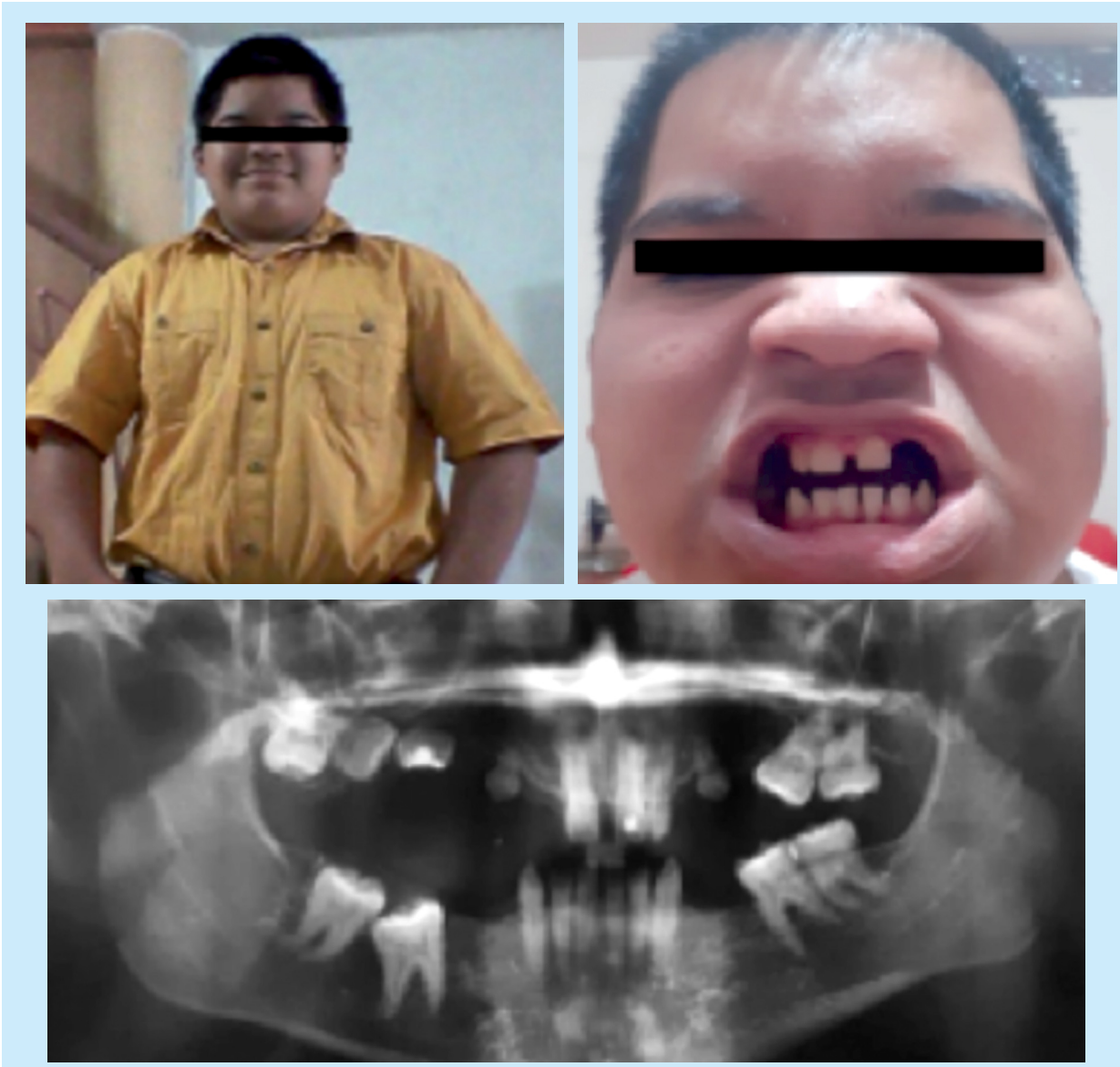
Figure 1. Characteristic fascie and dental radiography



the first finger of the two hands being substantially small by far, characteristic corresponding to type E2 brachydactyly. The X-ray shows marked metacarpal shortening and that of the distal phalanx of the thumbs (Figure 2). In addition, subcutaneous calcifications are detected radiologically in the posterior thorax and dorsum of feet.

The pubertal stage was Tanner V: penis length, 15 cm (normal >15 cm); testicular volume, 30 ml bilaterally (normal >20 ml).

Chvostek and Trousseau signs were positive, with total calcium levels: 1.67 mmol / L (normal value: 2.12-2.57 mmol/L); serum phosphorus: 1.81 mmol/L (normal value: 0.8-1.6 mmol/L); parathyroid hormone (PTH): 12.05 pmol/L (normal value: 1.5-8.97 pmol/L), and total vitamin D (25-OH-D): 76.38 nmol/L (normal value: 25- 137 nmol/L).

The biochemical evaluation established that the seizures, paresthesia, and Chvostek and Trousseau signs were attributed to hypocalcaemia. He was treated with an intravenous calcium infusion and later referred to the endocrinology department for a comprehensive evaluation.

In dual energy X-ray absorptiometry (DXA) bone densitometry, with DEXXUM-T equipment (OsteoSys - Seoul, Korea), he presented a decrease in bone mass for his age and gender in the lumbar area (L1-L4) , with preservation of bone mass in the femoral neck (lumbar spine: 1.044 g/cm^2, Z-score: -2.6; and femoral neck: 1.146 g/cm^2, Z-score: 0.3).

The computed tomography of the skull revealed cortical and subcortical calcifications, peri-ventricular, nuclei of the base and in the cerebellum. The nuclear magnetic resonance study with contrast medium showed periventricular calcifications.

The results of the current hormonal and biochemical determinations are presented in table 1. Total vitamin D (25 hydroxyvitamin D) was determined by electrochemiluminescence (normal value: 25-137 nmol/L). PTH was determined by chemiluminescence (normal value: 1.5-8.97 pmol/L). The determination of 1.25 dihydroxyvitamin D and PTH-induced cAMP was not carried out due to unavailability of hospital tests and financial constraints.

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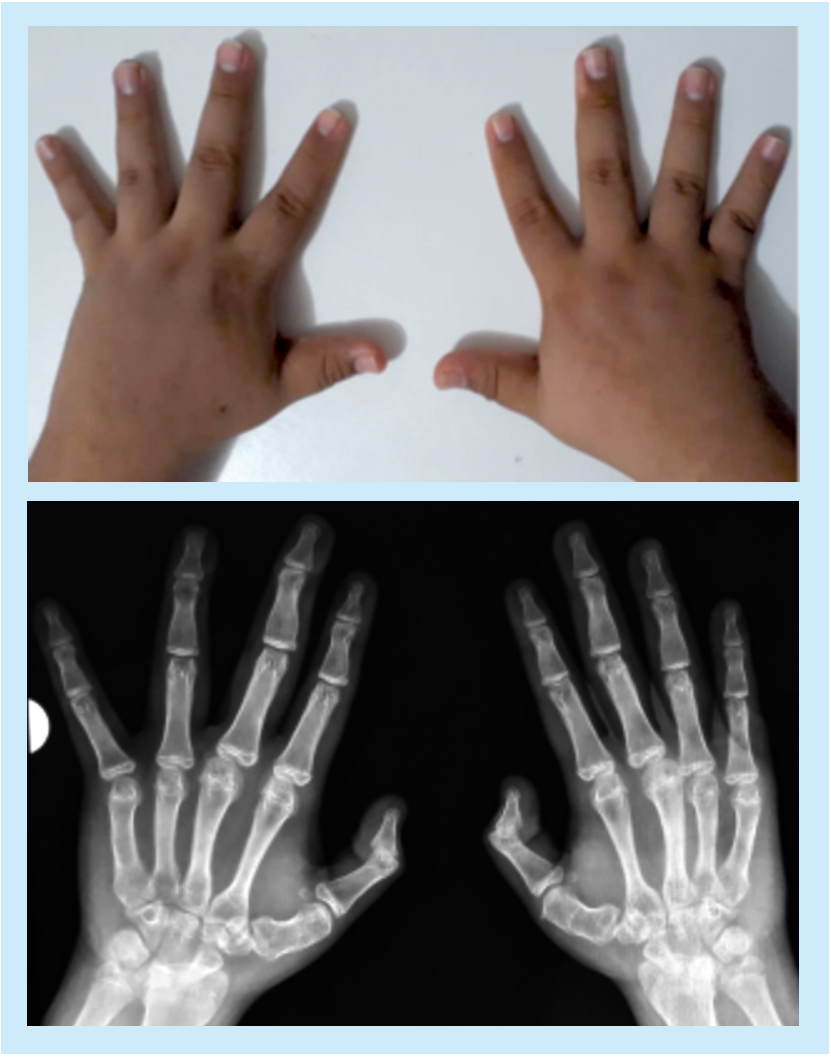
The complete blood count, blood glucose levels and liver and kidney function tests were normal. The biochemical and hormonal study of his mother shows normal values: calcium 2.35 mmol/L, phosphorus: 1.13 mmol/L, TSH: 3.5 mU/L, free T4: 14.1 pmol/L, PTH: 2.58 pmol/L.

At follow-up, serum calcium levels and urinary calcium/creatinine ratio were monitored to achieve optimal serum calcium levels.

The patient is currently being treated with oral calcium carbonate supplements, 3 g/day; oral calcitriol, 1.5 mg/day; vitamin D3, 2,000 IU/day, and levothyroxine, 150 µg/day. He attends outpatient check-ups every 3 months were he is taken measurements of serum calcium and phosphorus, PTH, vitamin D and thyroid hormones. In addition, he regularly attends the Neurology Service to control his seizures and the Psychology Department to get support for him and his family. The Nutrition Service offers dietary advice, and due to her dental alterations he is under permanent dental treatment.

Figure 2. Photograph and radiography of the hands. Marked shortening of the metacarpal bones and distal phalanx of the thumbs



Discussion

PHP is a clinically dysmorphic syndrome characterized by skeletal and developmental defects[11], including short height, rounded face, short fourth metacarpal bones as well as other bones in the hands and feet, obesity, dental hypoplasia, and soft tissue calcifications or ossifications[12,13]. However, some cases may present unusual phenotypic characteristics[12,13]. The biochemical characteristics of patients with PHP are hypocalcaemia, hyperphosphatemia and elevated levels of PTH[12].

In the present case, the diagnosis of pseudohypoparathyroidism was considered given the laboratory results compatible with resistance to PTH (hypocalcaemia, hyperphosphatemia and elevated PTH), together with the phenotypic characteristics of Albright's hereditary osteodystrophy[14] (AHO), which guided us towards a 1a or 1c PHP type.

Hypocalcaemia is a consequence of PTH bone resorption response loss, resulting in a defective mobilization of calcium from the bone and a lower absorption of calcium in the intestine[12].

Brachydactyly, described as shortening of the III-V metacarpal/metatarsal bones and the distal phalanx of the first finger, is one of the most specific characteristics of the Albright phenotype[14]. From the phenotypic characteristics of AHO, we highlight PHP type E brachydactyly; and this patient presents significant shortening in the metacarpus and distal phalanx of the thumb in both hands that could be considered a type E2 brachydactyly[14,15].

There is an association of PHP with variable resistance to multiple hormones that act through the Gsα protein. Resistance to TSH is the hormonal alteration that has been most commonly associated and can even be diagnosed before the appearance of phosphocalcium metabolism disorders[16]. In this case, the absence of antithyroid antibodies supports the resistance to TSH diagnosis[11].

Reproductive dysfunction has been associated with 1a PHP; however, the effects of hypogonadism are less evident in men[16]. The normal secondary sexual characteristics and the determination of sex hormones rule out the possibility of alteration in the gonadal axis in our patient.

There are discrepancies on the effects of PHP on the skeleton[8,17,18]. Some studies have reported that bone density is reduced in patients with PHP[17]. However, Long et al. analysed the bone mineral density in 22 subjects with 1a PHP and found that bone mass was normal or increased in all the studied bone regions[18]. On the contrary, in this case the bone mineral density measured in the lumbar region shows decreased values compared to the controls of same age and gender, with preservation of bone mass in the femoral neck.

Between 2000 and 2019, the international literature described approximately 325 cases of PHP[4,16,17]. A series

Table 1. Hormonal and biochemical determinations

Laboratory determinations	Results	Units I.S.	Normal values
Albumin	4.00	g/L	35-50
Anti-thyroid peroxidase antibodies	35.00	IU/ml	≤35
Urine calcium	1.12	mmol/day	<7.5
Total serum calcium	1.67	mmol/L	2.12-2.57
Calcitonin	3.60	ng/L	<100
Creatinine	35.36	umol/L	≤106
Serum phosphorus	1.81	mmol/L	0.8-1.6
FSH	1.70	IU/L	2-15
Free T4	14.16	pmol/L	10-23
Glucose	4.88	mmol/L	<6.1
HbA1c	4.60	%	<5.7%
HOMA IR	2.30	--	2.1-2.7
Plasma insulin	82.80	pmol/L	36-179
LH	1.83	IU/L	3-25
Serum potassium	3.93	mmol/L	3.5-5.3
PTH	12.05	pmol/L	1.5-8.97
Urinary D-Pyrilinks	6.60	nM DPD/mM Creatinine	(2.3-5.4)
Serum sodium	138.00	mmol/L	135-146
Testosterone	9.53	mmol/L	6.7-28.9
TSH	3.40	mU/L	0.5-4.70
Total vitamin D*	76.38	nmol/L	25-137

*: 25 hydroxyvitamin D.

of 60 PHP cases was published in Denmark in 2016, but only 30 (50%) of them underwent genetic testing for PHP, of which in 14 a mutation in the GNAS1 gene was identified. In those which could not be genetically confirmed (76%), characteristic biochemical and hormonal criteria were accepted as diagnosis, excluding cases with confirmed evidence of kidney failure, vitamin D deficiency, or any other known cause of secondary hyperparathyroidism[17]. In 2013, in a series of 72 cases with PHP treated in the Spanish National Health System, genetic confirmation could be made in 63 of the cases (88%)[16].

In Latin America, after a search throughout the literature ranging from the years 1957 and 2020, we found 32 publications, in which 56 cases of PHP are reported. Genetic studies in order to confirm the diagnosis were performed in only 6 of these publications. In most cases the diagnosis was based on the biochemical/hormonal and phenotypic profile.

There are no previously reported cases in our country, which is possibly due to subdiagnosis. However, data published worldwide describe the phenotypic characteristics associated with the biochemical alteration compatible with the findings in our patient. In our case, the clinical diagnosis of PHP could not be confirmed by a genetic study, but, as it is accepted in the literature, clinical and biochemical evidence are sufficient to precise the diagnosis of PHP[4,11].

In accordance with international criteria[12,15], our aim in long-term treatment has been to reduce the serum PTH level to the upper level of the reference range with 1-25 dihydroxyvitamin D and oral calcium, to improve calcium reabsorption in the distal renal tubule, prevent hypercalciuria and avoid alterations in bone mineralization[18].

Conclusions

In Latin American countries in which genetic studies are not available, we must bear in mind that, when presented with a patient with severe and persistent hypocalcaemia associated with high PTH, normal kidney function and a characteristic phenotype, the suspicion of a PHP must arise, even despite the lack of genetic confirmation.

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

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REVIEW
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Osteocalcin: from marker of bone formation to hormone; and bone, an endocrine organ

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Summary
Osteocalcin is a protein synthesized by the osteoblast. Before being released into the extracellular matrix, human osteocalcin undergoes gamma-carboxylation, as gamma-carboxy-glutamic acid binds at positions 17, 21 and 24. Part of the carboxylated and decarboxylated osteocalcin passes into the circulation. Since its discovery in the late 70s, it has been used as a marker of bone formation as it is an osteoblastic product and its role in the body is unknown. In recent years, osteocalcin has been identified as a hormone. Bone is considered an endocrine organ. Osteocalcin acting as a hormone is the decarboxylated form. Osteocalcin is involved in glucose homeostasis, skeletal muscle function, brain development, male fertility, hepatic steatosis, and arterial calcification. All of these facts have actually been tested in mice, but there is strong evidence that this could occur in humans. We are faced with facts that, if proven, would have enormous clinical significance.

Key words: osteocalcin, hormone, glucose, insulin, skeletal muscle, brain development, hepatic steatosis, arterial calcification.

Osteocalcin is a protein synthesized by the osteoblast. It was identified in the late 1970s and in humans contains 49 amino acids[1]. Before being released into the extracellular matrix, osteocalcin undergoes gamma-carboxylation, as gamma-carboxy-glutamic acid binds at positions 17, 21 and 24. A gamma-carboxylase is involved in this reaction and the presence of vitamin K is required (Figure 1). The presence of the two carboxyl groups causes gamma-carboxylated osteocalcin to have a high affinity for calcium and, when released into the extracellular environment, binds in a large proportion to hydroxyapatite in bone. A part of this gamma-carboxylated osteocalcin and also non-carboxylated osteocalcin remain in the circulation[2]. Only 10-30% of the synthesized osteocalcin reaches the circulation, and the rest remains attached to the bone matrix. Non-carboxylated osteocalcin represents 1/3 of total osteocalcin. During resorption, when the bone matrix is destroyed, part of the osteocalcin that is bound to the bone passes into the circulation[2]. Osteocalcin is only synthesized by osteoblasts and is the most abundant non-collagenous protein in the extracellular matrix and is the tenth most abundant protein in vertebrates[3]. Since first reported, its levels were correlated with bone formation[4]. For all researchers working in bone metabolism, having a new bone formation marker was a breakthrough when the only markers of remodeling that were available up to that time were hydroxyproline and total alkaline phosphatase. The bone isoenzyme of alkaline phosphatase could also be measured by a rather complex method by electrophoresis. Osteocalcin has been used for many years as a marker of bone formation in practically all the work carried out in this regard. It is used less since 2011 when the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) recommended that the N-terminal propeptide of type I collagen (PINP) be used as a marker of formation and the C-terminal β-telopeptide of type I collagen or β-crosslaps (β-CTX) as a marker of resorption in clinical studies on osteoporosis[5].

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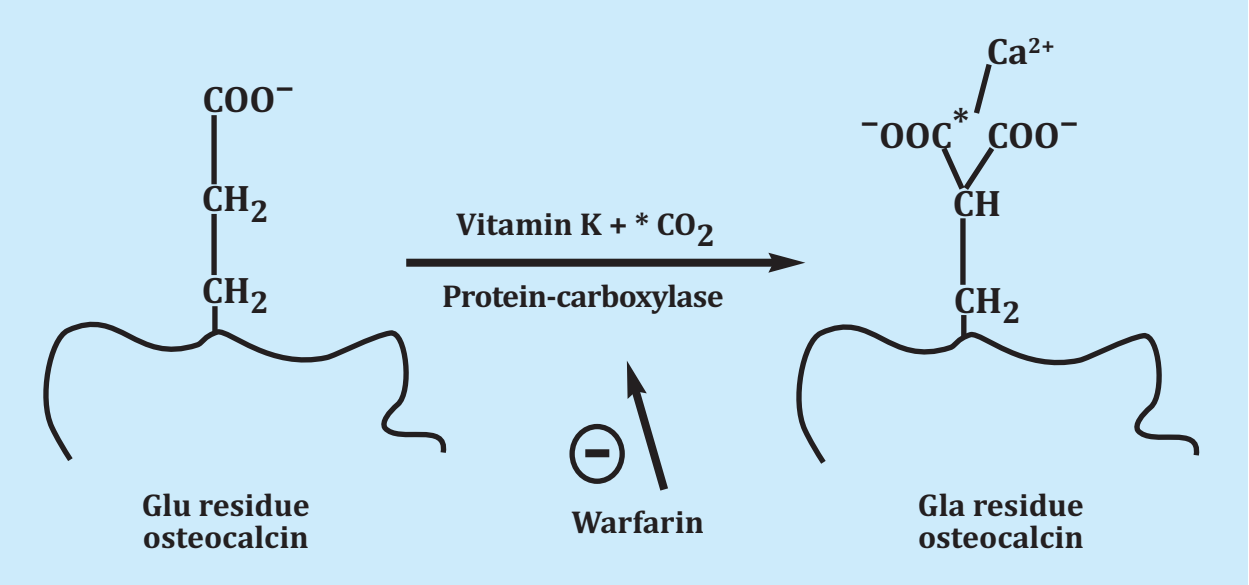
Figure 1. By post-translational carboxylation, the glutamic acid of osteocalcin is transformed into gamma-carboxyglutamic acid. The presence of the two carboxyl groups causes a high affinity for the calcium Ca^{2+} binding of the extracellular matrix



For many years, and despite its abundance, the role of osteocalcin in the body was unknown. Due to its post-translational modification, it was thought to be involved in bone mineralization. But when mice without osteocalcin (osteocalcin-/-) were obtained, it was found that these mutants presented totally normal mineralization, making it clear that the function of osteocalcin was not related to bone mineralization[6].

Bone contains osteoclasts, cells whose function is to destroy bone, and this active destruction of mineralized bone requires energy[7]. Bone formation also requires energy[8]. Thus, researcher groups such as Karsenty et al. hypothesized that bone modeling and remodeling must be associated with the regulation of energy metabolism[9,10]. Probably, the amount of energy from the active destruction of the bone is proportional to the surface occupied by it. This energy requirement is probably very high, since bone resorption does not occur as an isolated event, but in the context that there must be a coordinated regulation of a biphasic function called modeling during childhood and remodeling in adult life. This is why the aforementioned authors hypothesized that bone modeling and remodeling must be linked to the regulation of energy metabolism. This vision of a coordinated regulation of bone mass and energy metabolism is supported by clinical evidence. For example, longitudinal bone growth stops in children and bone mass declines in adults with severely limited access to food (ie, energy)[11]. Furthermore, another link between bone remodeling and energy metabolism is that bone mass always declines in both sexes when gonadal function decreases. Considering these observations, it is concluded that there must be a coordinated regulation of bone growth/mass, energy metabolism and reproduction[9].

We previously mentioned that mice (osteocalcin-/-) had normally mineralized bones, but they develop some phenotypes that can only be explained, given the site of synthesis of osteocalcin and its abundance, if this molecule were acting as a hormone. Indeed, the mutant mice (osteocalcin-/-) had more visceral fat than the controls and also had fewer offspring. The systematic study of these phenotypes established that bone must be an endocrine organ and that the hormone it secretes, osteocalcin, affects energy metabolism and fertility. In other words, there is a coordinated regulation, endocrine in nature of energy metabolism and reproduction. A fundamental part is that the bone would be an endocrine organ and not the receptor of the action of hormones.

At this time, the knowledge of the mechanisms of action of osteocalcin in its target organs is a work in progress. For example, increased adiposity in mice (osteocalcin-/-) could be associated with a decrease in energy expenditure and not an increase in appetite (another function regulated by bone). The molecular basis of this phenomenon has not yet been discovered[10].

Osteocalcin and glucose homeostasis

In a study by Wei et al.[9], in which they cultured mouse osteoblasts with pancreatic islets, increased insulin expression in the islets was observed. Osteoblasts did not increase the expression of any other hormones secreted by the pancreatic islets. When osteoblasts from mice (osteocalcin-/-) were added, this insulin expression did not occur. Thus, it was shown that osteoblasts are endocrine cells that regulate insulin expression and that osteocalcin is the hormone responsible for this action. Mice (osteocalcin-/-) on a normal diet were also found to be hyperglycemic and hypoinsulinemic. Insulin secretion was decreased in the absence of osteocalcin.

In this same study, a glucose tolerance test showed that the mice (osteocalcin-/-) were glucose intolerant because they had a decrease in insulin expression. Wei et al.[9] stated that the fact that osteocalcin regulates glucose metabolism is not synonymous with bone being the origin of diabetes. It simply increases the regulatory landscape of glucose metabolism.

Research carried out in rats with normal diet shows that osteocalcin is necessary and sufficient to promote the proliferation of β cells in the pancreatic islets, to promote the expression and secretion of insulin and to promote glucose uptake in peripheral tissues. and hence glucose homeostasis[12].

The receptor that mediates the osteocalcin signal in β-pancreatic cells and other peripheral tissues is a G-protein coupled receptor called GPR6a[13]. Specific gene deletion and other genetic experiments have established that, *in vivo*, osteocalcin is, without a doubt, the ligand that explains the regulation of glucose homeostasis through GPR6a[10]. The biological importance of the regulation of osteocalcin in glucose homeostasis has been verified in normal mice fed a high fat diet. Exogenous osteocalcin almost completely rescues glucose intolerance in these animals[14].

It was very important to know if these actions were produced by carboxylated or decarboxylated osteocalcin. In bacteria there is no gamma-carboxylation and thus recombinant bacterial osteocalcin that was not gamma-carboxylated could be produced. This non-carboxylated osteocalcin was able to induce insulin expression in the pancreatic islets, indicating that the non-carboxylated form of osteocalcin is the one that acts as a hormone[9].

Osteoblasts have insulin receptors on their surface, and it is of great interest that insulin and osteocalcin are involved in a regulatory loop. So the insulin signal in the osteoblasts is required for good glucose homeostasis throughout the body[9].

Mice that do not have insulin receptors on osteoblasts, when eating a normal diet, experience a decrease in the active circulating form of osteocalcin, a decrease in insulin secretion, glucose intolerance and insulin resistance. The insulin signal on osteoblasts inhibits the expression of osteoprotegerin and favors bone resorption. The low pH that occurs under osteoclasts favors the formation of decarboxylated osteocalcin, which is the active form of osteocalcin. This active osteocalcin acts again on the β-pancreatic cells and new insulin is formed that acts again on the osteoblast and the cycle begins again (Figure 2)[15].

Osteocalcin and skeletal muscle

A simple injection of exogenous osteocalcin immediately before exercise or chronic administration of this hormone for 1 month, not only increases the exercise capacity of young mice but also restores the exercise capacity of older mice[16]. At the same time as increasing muscle strength in aged mice, chronic administration of osteocalcin promotes muscle mass gains[17]. That is, exogenous osteocalcin is not only necessary but sufficient to reverse the decline in exercise capacity and muscle mass observed in older mice[18].

Osteocalcin signal on skeletal muscle is carried out through the GPR6a receptor[18]. Osteocalcin has been shown to regulate nutrient uptake and catabolism in muscle during exercise. Glucose, the main nutrient used by skeletal muscle to generate energy during exercise, is stored in myofibers as glycogen. The degradation of glycogen in skeletal muscle during exercise is lower in mice that do not have the GPR6a receptor and in mice (osteocalcin-/-), showing that osteocalcin favors glycogenolysis.

It has also been observed that the accumulation of tricarboxylic cycle intermediates in skeletal muscle seen in mice after exercise is not observed in mice (GPR6a-/-), indicating that there is no ATP input from the tricarboxylic cycle. Over an extended period of exercise, when animals deplete their glycogen stores, the uptake and catabolism of fatty acids increases in skeletal muscle[19]. Osteocalcin favors the oxidation of fatty acids in myofibers. The osteocalcin signal in the myofibers promotes the uptake and catabolism of glucose and fatty acids during exercise, which is why a decrease in physical activity is observed in mice (osteocalcin-/-) and (GPR6a-/-) when compared to controls[20].

For decades it has been common knowledge that exercise induces changes in the immune system[21]. Interleukin 6 (IL6) was the first molecule that was seen to be released into the blood in response to muscle contraction[22]. IL6 promotes glucose uptake and fatty acid oxidation in skeletal muscle, increasing glucose production in the liver and lipolysis in adipose tissue[23].

Exercise induces bone resorption and the production of bioactive osteocalcin. Mice (IL6-/-) after exercise do not increase bone resorption markers or bioactive osteocalcin, as happens in healthy mice. This suggests the existence of a loop between bone (via osteocalcin) and muscle (via IL6) that promotes adaptation to exercise[20]. Thus:

- Osteocalcin increases the uptake of nutrients and their catabolism in skeletal muscle.
- Osteocalcin increases the secretion of IL6 in skeletal muscle. This leads to the generation of extra-muscular glucose and fatty acids[24].
- IL6 increases the production of bioactive osteocalcin[20].

IL6 and osteocalcin regulate similar aspects of skeletal metabolism during exercise, increasing glucose and fatty acid catabolism.

But, does osteocalcin act independently of IL6 in these processes? Mera et al.[16] studied the action of osteocalcin on a myofiber culture in normal mice and mice (IL6-/-). Osteocalcin induced these functions in the two types of culture, which reveals that IL6 is not required for the catabolic action of osteocalcin in skeletal muscle.

The identification of a bridge between bone, via osteocalcin, and skeletal muscle, via IL6, which is necessary and sufficient to promote adaptation to exercise in young and old animals, represents a significant advance in our understanding of how the skeletal muscular system controls a function that is essential for the survival of all vertebrates[20].

Osteocalcin and brain development

Osteocalcin is necessary for the development of the brain and for its function. Its absence in mice produces a profound deficit in spatial knowledge and memory and an exacerbation of anxiety. Ostecalcin could prevent lowering of consciousness due to age[25].

The brain-derived neurotrophic factor BDNF, a well-known molecule that participates in memory, dependent on the hippocampus, is the mediator of the regulation of osteocalcin on cognitive function[26]. Non-carboxylated osteocalcin is able to stimulate the dynamics of BDNF vesicle transport towards sypnasis in rat neurons[25].

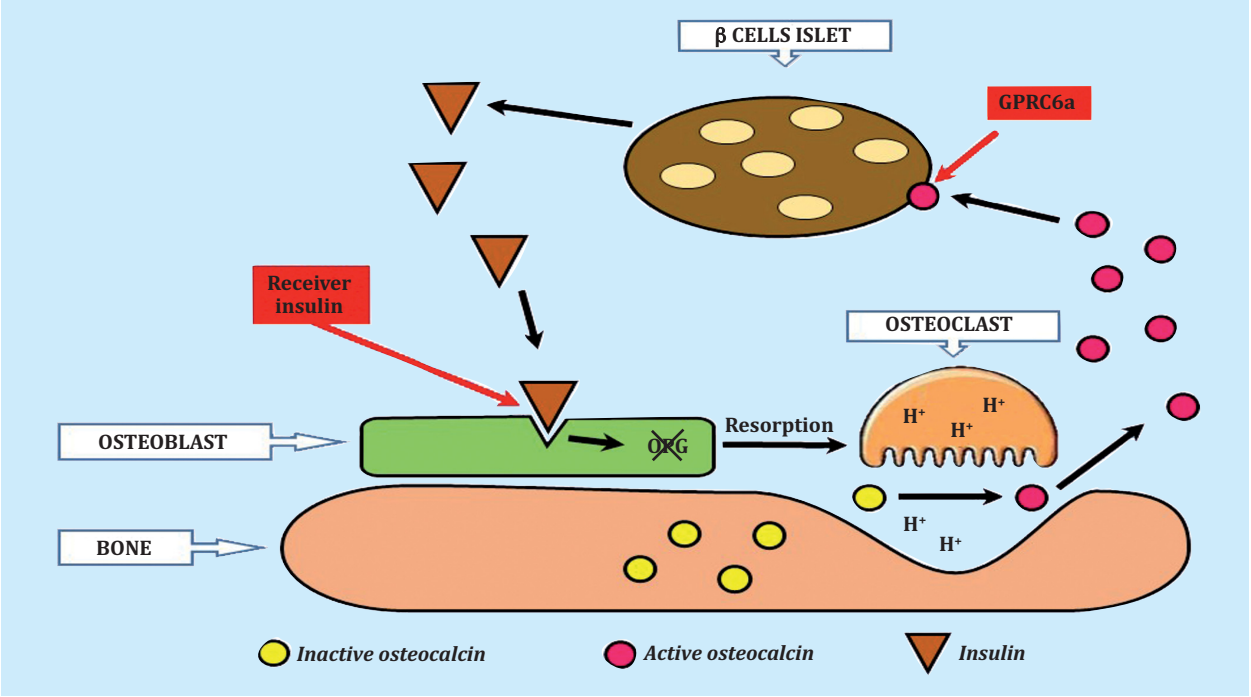
Khrimian et al.[27] identified the receptor that transfers the signal from osteocalcin to neurons. It is Gpr158, a G protein-bound receptor expressed on neurons in the CA3 region of the hippocampus, which transmits the osteocalcin signal through inositol 1,4,5-triphosphate and a brain-derived neurotrophic factor. This is very important for future therapeutic actions.

Osteocalcin and male fertility

Decarboxylated osteocalcin acts on the Leydig cells of the testes, promoting the biosynthesis of testosterone[28]. Decarboxylated osteocalcin acts through the GPRC6a receptor, as it did on β-pancreatic cells.

Osteocalcin has also been shown to act via a pancreas-bone-testes axis that regulates, independently and in parallel to the hypothalamic-pituitary-testes axis, male reproductive function, promoting testosterone biosynthesis[29].

Figure 2. Osteocalcin-insulin cycle. Decarboxylated osteocalcin, the active form, binds to the GPRC6a receptor on pancreatic ß cells, causing the release of insulin. Insulin then binds to the insulin receptor on the osteoblast, causing a decrease in osteoprotegerin synthesis and an increase in bone resorption. In the acidic environment of the osteoclast, the inactive, gamma-carboxylated osteocalcin becomes active, non-carboxylated. Active osteocalcin restarts the cycle by binding to the ß cells of the pancreatic islets



Based on Wei et al.[9]

Osteocalcin and hepatic steatosis

In mice on a high-fat diet, daily injections of osteocalcin of 3 or 30 ng/g of body weight/day partially restore insulin sensitivity and glucose tolerance. Furthermore, mice treated with intermittent injections of osteocalcin exhibited increased energy expenditure and were protected from diet-induced obesity. Finally, the fatty diet-induced hepatic steatosis was completely avoided in the mice that received osteocalcin daily. These results show that daily ostecalcin injections improve glucose management and prevent the development of type 2 diabetes[14].

Osteocalcin and arterial calcification

In the "bone-vascular calcification paradox" there is high calcification in the vessels, leading to arterial stiffness and cardio-vascular disease, and reduced calcification in the bone leading to osteoporosis and bone fracture. This leads to the hypothesis that bone metabolism and cardiovascular disorders could have common pathogenic pathways, leading to the expression “bone-vascular axis”[30]. Several molecules seem to play a role in this axis, and one of them would be osteocalcin.

Rashdan et al. maintain the hypothesis that osteocalcin regulates the calcification of vascular smooth muscle cells[31]. Immunohistochemistry reveals the co-localization of osteocalcin with calcification of vascular smooth muscle cells in calcified plaques of carotid arteries. Osteocalcin involvement in the development of arteriosclerosis is supported by a recent meta-analysis by Millar et al., in which a relationship between osteocalcin and atherosclerosis markers is observed in histological samples[32]. In this same study, the authors found no differences between osteocalcin levels in patients with and without vascular events. That is, osteocalcin seems to be a marker only of the calcification process.

Osteocalcin in humans

So far, all the review carried out on the role of osteocalcin as a hormone has focused on experiments in mice or rats. It is extremely important to know if these facts can be transferred to humans, with the clinical implications that this would entail.

A systematic review of the literature conducted between 2007 and 2014 identified 82 studies that observed that serum levels of decarboxylated or total osteocalcin are negatively correlated with blood glucose, insulin resistance, obesity, or markers of metabolic syndrome. Furthermore, some of the human data support a role for osteocalcin in insulin secretion[33].

Treatment with bisphosphonates has been found to decrease non-carboxylated osteocalcin in serum and that levels of it and/or markers of insulin sensitivity or secretion are positively correlated with markers of bone resorption in humans[34].

It has also been seen that patients with a dominant form of osteopetrosis due to a defect in osteoclast activity are characterized by decreased levels of decarboxylated osteocalcin and hypoinsulinemia[15].

Changes in osteocalcin levels, following bisphosphonate treatments, are associated with changes in body mass and fat[35].

Oury et al. analyzed a cohort of patients with testicular failure and identified 2 individuals with a variant in one of the GPRC6a domains. These patients had glucose intolerance and insulin resistance[36].

Osteocalcin levels have been compared between patients with type 2 diabetes mellitus and the non-diabetic population, with diabetics having lower levels of osteocalcin[37]. Patients with metabolic syndrome also have lower levels of total osteocalcin than healthy individuals.

In addition, there is a correlation between total and decarboxylated osteocalcin with markers of glycemic status and other cardio-metabolic parameters[38]. These authors point out the need to delve into these findings and their possible participation in human health, as well as analyze their possible therapeutic potential.

An observational study assessed the association between serum levels of osteocalcin and knowledge capacity in healthy adults, showing that it is positively correlated with measures of global knowledge in elderly women[39]. In children and adolescents with nonalcoholic fatty liver, the concentration of osteocalcin is inversely correlated with liver enzymes and with the severity of the disease[40].

Smith et al., in a study carried out in 2020[41], considered the normal values of decarboxylated and carboxylated osteocalcin in adult men. These values should be included in future studies in clinical trials and associated with the prediction of events such as fractures or risk of diabetes. The amount of evidence on the multi-organ effects of decarboxylated osteocalcin, supported by the facts demonstrated *in vivo* and *in vitro*, indicates the need to deepen these findings and its possible participation in human health, as well as to analyze its possible therapeutic potential.

Conclusions

The discoveries made in recent years concerning the role of osteocalcin as a hormone and bone as an endocrine organ are truly surprising. Osteocalcin, which for bone metabolism researchers was simply a marker of bone formation with no known function.

In mice, osteocalcin is involved in glucose homeostasis, skeletal muscle function, brain development, hepatic steatosis, male fertility, and arterial calcification.

We begin to find works that seem to anticipate that this could also happen in humans.

It is extremely important to address this type of research in humans, because if what happened in humans were similar to what happens with osteocalcin in mice, the therapeutic implications of this compound would be extremely interesting.

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

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SPECIAL ARTICLE
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Connexin 43 and cellular senescence: new therapeutic strategies for treating osteoarthritis

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Osteoarthritis (OA) is one of the most prevalent rheumatic diseases at present. It is characterized by the progressive degeneration of articular cartilage accompanied by alterations in other tissues, such as in the subchondral bone, synovial tissue or muscle. Currently one of the most frequent causes of disability in the aging population worldwide, OA is one of the main causes of chronic pain. From the biomechanical point of view, the joint is involved in maintaining mechanical support by stabilizing movement and flexion. The mechanical consequences of joint degeneration include the loss of stability or increased load stress on the joints, associated with changes in the structure and composition of the articular cartilage. Given that the molecular mechanisms by which joint tissue degradation and the loss of its homeostasis occur are not yet known, the current treatments available are based on the use of anti-inflammatories and pain relief drugs.

Articular cartilage is a tissue with unique mechanical properties formed by a dense extracellular matrix (ECM) that covers the surface of the bone in mobile joints, mainly composed of different types of collagen, proteoglycans and glycoproteins. Chondrocytes, the only cell type described in articular cartilage, are the cells responsible for synthesizing ECM components, as well as maintaining tissue homeostasis. Taking into account the distribution of chondrocytes within cartilage, until a few years ago it was believed that chondrocytes were found in isolation in gaps inserted in the ECM without any type of cellular interaction or communication between them. However, recent results have shown that chondrocytes present cytoplasmic projections that are capable of crossing the ECM and connecting distant cells[1]. In line with these results, it has been shown that chondrocytes express several proteins of the connexin family, involved in cellular communication through gap junctions (GJs). In the case of cartilage, chondrocytes are capable of communicating through connexin channels formed mainly by connexin 43 (Cx43)[2]. Furthermore, through these cytoplasmic projections and gap junctions, chondrocytes are capable of exchanging different metabolites and small molecules such as ATP or RNA in addition to amino acids and proteins[1,3]. On the other hand, several studies indicate that the overactivity of Cx43 triggers an inflammatory and degenerative process related to joint degradation in patients with OA[2]. In our research group we have shown that alterations in Cx43 activity trigger changes in the phenotype of chondrocytes accompanied by an increase in the expression levels of interleukin-1β (IL-1β), cyclooxygenase-2 (COX-2) and metalloprotease-3 (MMP-3)[4] associated with the progress of the disease. The overexpression of Cx43 in a chondrocyte line increases the CD105 and CD166 markers associated with de-differentiated stem cells, as well as the translocation to the nucleus of the Twist-1 transcription factor, which indicates that they could be undergoing a process of epithelium-mesenchyme transition (TEM)[4]. Lastly, Cx43 overactivity is associated with increased levels of senescence markers such as p53, p16 and ß-galactosidase, as well as activation of NF-κβ accompanied by a senescent phenotype and increased secretion of inflammatory cytokines, known as the secretory senescence-associated phenotype (SASP)[4]. These results show that alterations in the expression and activity of Cx43 could be playing an essential role in the development and progression of the disease by modulating the phenotype of the adult chondrocyte. In fact, the decrease in Cx43 activity using different compounds improves the regeneration capacity of different tissues and in different models of age-associated diseases[5,6], reinforcing the role of this transmembrane protein in tissue degeneration and senescence.

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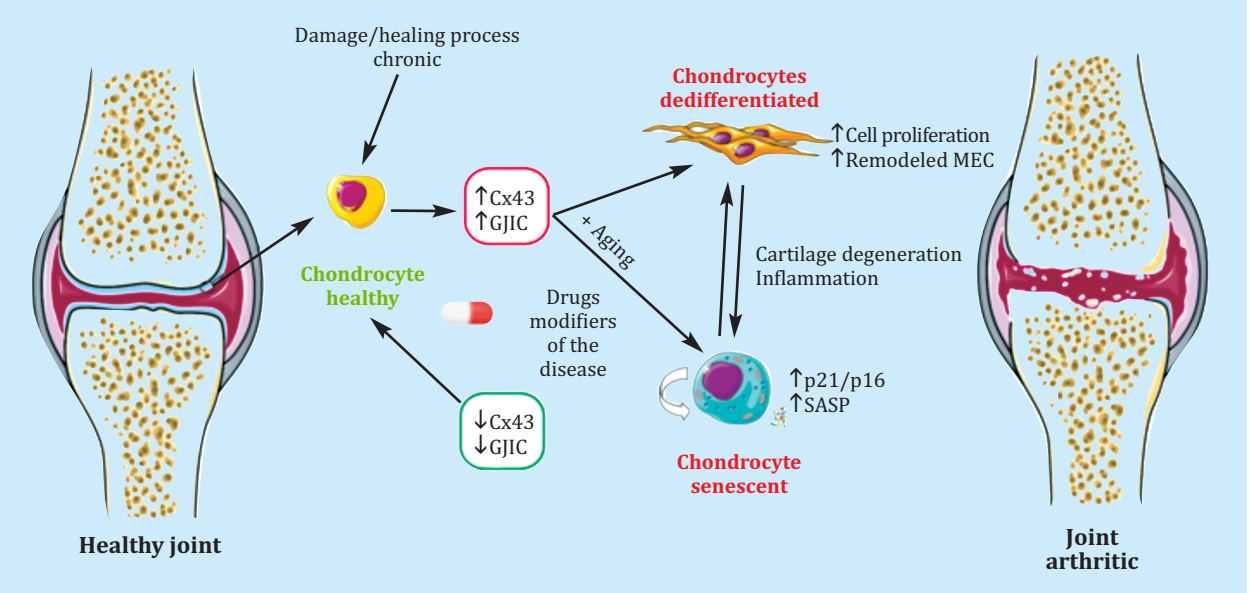
Figure 1. Cx43 is involved in the processes of de-differentiation and senescence in chondrocytes associated with inflammatory and degenerative processes in patients with OA. Decreasing Cx43 levels with osteoarthritis modifying drugs (DMARDs) could reverse this process and favor a regenerative environment that would prevent the progression of the disease



More studies are undoubtedly needed in this regard, but with our results, we could conclude that Cx43 is a therapeutic target of interest to maintain the adult chondrocyte phenotype, and avoid processes of de-differentiation and cellular senescence associated with an inflammatory and degenerative phenotype when it is maintained over time (chronically). In fact, *in vitro* models have already demonstrated its usefulness in reducing cell senescence markers and favoring chondrocyte re-differentiation, restoring tissue regeneration capacity[7-9]. In older adults, it should be noted that recently obtained results by our research group indicate that the increase in Cx43 could also be involved in tissue degeneration and accumulation of senescent cells in cases of intervertebral disc degeneration, suggesting that therapies aimed at modifying Cx43 They could be useful in the treatment of degenerative conditions in the intervertebral disc.

In recent years, different OA modifying drugs have been proposed as new therapeutic strategies because of their ability to promote chondrogenesis, thus promoting re-differentiation of chondrocytes and improving tissue regeneration. On the other hand, molecules capable of reducing Cx43 levels, such as oleuropein[10], improve ECM formation in 3D models by increasing levels of type II collagen and proteoglycans, and also improve the arthritic chondrocyte phenotype by reducing gene expression levels inflammatory interleukins and metalloproteases[10,11].

These and other studies show that high levels of Cx43 in cartilage observed from the first stages of the disease could be related to the activation of degradation processes of articular cartilage by activating the epithelium-mesenchyme transition (cell de-differentiation) and increasing cell senescence synergistically (Figure 1). Undoubtedly, the use of molecules and compounds that decrease the levels or activity of this protein will be of interest for developing new therapeutic strategies for the treatment of degenerative musculoskeletal diseases associated with age, such as osteoarthritis.

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COVID-19 and vitamin D. Position paper of the Spanish Society for Bone Research and Mineral Metabolism (SEIOMM)

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Introduction

Vitamin D exerts its effect mainly through its active metabolite, 1,25-dihydroxycholecalciferol, by binding to a receptor with wide distribution in the different cells of the body. This receptor regulates the expression of genes involved in different biological functions, including organ development, cell cycle control, phosphocalcic metabolism, detoxification, and control of innate and adaptive immunity[1,2]. Regulation of the vitamin D receptor is determined by interacting environmental, genetic, and epigenetic factors.

Vitamin D increases intestinal absorption and tubular reabsorption of calcium, inhibiting PTH synthesis. This leads to a reduction in bone turnover, which helps maintain its strength and reduce the risk of fractures. In addition, it exerts an intraosseous effect, facilitating the mineralization of the matrix, which prevents the development of rickets in children and osteomalacia in adults. Numerous studies have been published showing an association between low levels of vitamin D and various chronic diseases, such as cancer, diabetes, cardiovascular diseases, multiple sclerosis, and infectious diseases, among others[3]. These associations can be explained through different pathophysiological mechanisms related to vitamin D deficiency.

In 2020, the pandemic derived from COVID-19 occurred with a high rate of contagion and mortality. The seriousness of the process has made it necessary to apply therapeutic measures without clear scientific evidence[4]. Many of them have not proved effective in subsequent cohort studies and clinical trials of a different nature, so they have been withdrawn. Some have shown usefulness in certain periods of the disease.

Vitamin D is a hormone whose deficiency has been associated with numerous acute and chronic diseases, both bone and non-bone. However, the studies carried out to demonstrate the causality of the association, in general, have not been positive. The fact that various risk factors associated with the incidence and severity of COVID-19, such as north latitude, advanced age, non-Caucasian races, high blood pressure and diabetes, have also been associated with vitamin D deficiency[5], suggesting the possible link between COVID-19 infection and vitamin D deficiency.

This leads to the following questions:

- Is there a relationship between vitamin D deficiency and the risk of coronavirus infection?
- Is there a biological explanation for this association?
- Can the administration of vitamin D to deficient individuals prevent infection or alter its severity?
- What is the risk/benefit ratio of its administration?

Is there a relationship between vitamin D deficiency and the risk of SARS-COV-2 coronavirus infection?

Although not all the data are uniform, there does seem to be a relationship, not necessarily a causal one, between vitamin D deficiency and the incidence and mortality from COVID-19.

Is there a relationship between vitamin D deficiency and the risk of coronavirus infection?

Initially, mortality from COVID-19 was reportedly higher in northern latitudes, which could be attributed to decreased production of vitamin D due to the effect of ultraviolet radiation. However, Spain and Italy, located in southern Europe, presented a very high mortality, as well as a high prevalence of hypovitaminosis D[6]. Illie et al.[7] carried out an ecological study in 20 European countries. They found an inverse relationship of vitamin D levels with the incidence of COVID-19 (r:-0.443; p=0.05) and mortality due to disease (r:-0.4378; p=0.05). In another study carried out in 117 countries, an association between latitude and mortality was observed (p<0.033), after adjusting for age[8]. Meltzer et al.[9], in a 489-patient cohort, with 75% women, found that COVID-19 infection risk was associated with advanced age, non-Caucasian race and vitamin D deficiency. The risk of infection in individuals with vitamin D sufficiency was 12.2%, compared to 21.6% in those with insufficiency (p=0,02). D'Avalio et al.[10] reported that patients with positive PCR had vitamin D levels of 11.1 ng/ml, while, among those with negative PCR for COVID-19, the levels were 24.6

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ng/ml; p=0.004. Another study linked vitamin D levels with mortality, finding that patients with vitamin D below 10 ng/ml had a 50% chance of dying, compared to 5% of those with a higher level, although the study sample size was small[11]. Hernández et al.[12] found lower levels of vitamin D in hospitalized patients, unrelated to the severity of the disease, although they observed an inverse relationship with the levels of ferritin and D-dimer, both parameters related to the severity of the infection.

The relationship between low vitamin D levels and the risk of infection by COVID-19 has been observed in a recent meta-analysis[13]. Pereira et al.[14] conducted a meta-analysis that included 8,176 patients with COVID-19 infection. These authors did not find a relationship between vitamin D deficiency and an increased risk of infection, but did find a relationship with its severity. A study conducted in England with biobank samples also found no association between vitamin D and COVID-19[15]. It must be taken into account that, in critical patients, there is a high prevalence of vitamin D deficiency, although we do not know if it is an "innocent bystander", a marker of severity or a real and modifiable risk factor. The stimulation of renal 1α-hydroxylase in the face of inflammatory processes means that the association of various acute processes has the possibility of being an effect rather than a cause, with 25-hydroxyvitamin D levels being a negative acute phase reactant[16].

So, although the studies carried out have different approaches and their results are not uniform, in general an association, not necessarily causal, is observed between vitamin D deficiency and the incidence and mortality from COVID-19.

Is there a biological explanation for the association between vitamin D deficiency and incidence and mortality?

Vitamin D can play a protective effect thanks to:
- The maintenance of the integrity of the epithelium.
- The stimulation of the production of antimicrobial peptides.
- The reduction of the inflammatory response.
- Modification of the relationship between ACE/ACE2 by increasing the expression of ACE2.

Is there a biological explanation for the association between vitamin D deficiency and incidence and mortality?

Vitamin D may play a role in reducing the incidence and mortality of COVID-19 through various mechanisms, such as the maintenance of epithelial integrity, the production of antimicrobial peptides, the reduction of the inflammatory response and the modification of the relationship between ACE/ACE2 (classical angiotensin converting enzyme/angiotensin converting enzyme 2) by increasing ACE2 expression.

a) Epithelial integrity: vitamin D stimulates the expression of gap protein and tight junction protein that help to maintain the integrity of the epithelium, preventing the penetration of the virus. Furthermore, it acts indirectly by stimulating autophagy and facilitating the death of epithelial cells occupied by the virus. This effect is carried out through modulation of the mTOR metabolic pathway[17].

b) Production of antimicrobial peptides: 25-hydroxycholecalciferol is transformed into 1,25-dihydroxycholecalciferol (calcitriol) at the level of monocytes and macrophages that express CYP27B1 (1-α hydroxylase), and facilitates the development of antigen-presenting cells. Calcitriol stimulates the production of cathelicidin, defensin and NOD2 (nucleotide binding oligomerization domain-containing protein 2), facilitating the destruction of microorganisms. In addition, it increases the synthesis of hepcidin, which accumulates iron at the cellular level, preventing its use by microorganisms and stimulates the production of nitric oxide and superoxide. All these proteins have an antiviral action and are produced by stimulating innate immunity[18,19].

c) Stimulation of innate immunity, mediated by vitamin D, decreases the proliferation of types 1 and 17 helper T lymphocytes and increases that of helper 2 lymphocytes and regulatory T lymphocytes. The result is a decrease in pro-inflammatory cytokines (IL1, IL6, IL12, TNFα, IL17, and interferon γ) and an increase in anti-inflammatory cytokines (IL10). This decrease in inflammatory cytokines can be mediated through the metabolic pathway of NFkβ. All these effects modify acquired immunity[17,20].

d) The entry of SARS-COV-2 into the body's cells, and, therefore, the start of the infectious process, is carried out through the ACE 2 receptor. A paradoxical phenomenon occurs, since ACE 2 is expressed less intensely in men and the elderly who, on the other hand, are those who present a greater risk of serious infection by COVID-19[21]. Vitamin D is a potent renin inhibitor, so its administration facilitates a decrease in the classic ACE/ACE2 ratio that reduces cardiovascular morbidity and mortality.

All these facts represent the biological bases that could explain the possible beneficial effect of vitamin D.

Can administering vitamin D to deficient individuals prevent infection or alter its severity?

The evidence to indicate the administration of vitamin D in the prevention or treatment of COVID-19 is scarce and presents numerous limitations.
At this time, we do not know the vitamin D threshold that must be reached to achieve the objective, the most suitable metabolite or the doses to be used.

Can administering vitamin D to deficient individuals prevent infection or alter its severity?

A meta-analysis that included more than 11,000 patients, from 25 clinical trials, showed a beneficial effect of vitamin D in reducing infectious diseases of the respiratory tract. The effect was greater in situations with severe vitamin D deficiency (<10 ng/ml) and with daily or weekly administrations[22]. Taking into account these data, the existence of hypovitaminosis D in patients with COVID-19 and a biological explanation that offers plausibility to a beneficial effect, 18 clinical trials have been proposed that try to demonstrate this hypothesis[10]. The beneficial effects could take place both in the early viremic phases, preventing the development of the disease, and in late hyperinflammatory phases.

However, the evidence available so far is very scarce. Several case/control studies have been published that we can call quasi-experimental and a pilot study from a cohort of patients infected with pneumonia (Table 1). Their sample size is small, except for one of them, which included 1,476 patients. Some favorable results have

Table 1. Vitamin D-COVID-19 studies

Autor	Study types	Population (N)	Supplement	Objetive	Results	Comments
Fasano et al.[23]	Cases/Controls	Patients with disease of Parkinson's (1,486)	Not established	Incidence of COVID-19	12.4% vs. 22.9% (p=0.010)	Those who receive supplements have less incidence
Annaweiler C et al.[24]	Cases/Controls	Institutionalized (7)	Cholecalciferol 50,000 IU/month (prior) 80,000-100,000 IU/2-3 months (prior) 80,000 IU single bolus after diagnosis	Mortality	6.9% vs. 31.3% (p=0.017) 18.8% vs. 31.3% (p=0.5)	Those who receive vitamin D in the previous year have less mortality, but not those who receive it after diagnosis. The doses are higher than those usually recommended
Annaweiler G et al.[25]	Cases/Controls	Institutionalized (66)	Cholecalciferol bolus of 80,000 IU before or after diagnosis	Mortality	17.5% vs. 55.6% (p=0.023)	Those who receive vitamin D have less mortality. The doses are higher than those usually recommended
Tan CW et al.[26]	Cases/Controls	Hospitalized by COVID-19 (43)	Cholecalciferol (1,000 IU/day), magnesium, B12 vitamin	Mortality	17.6% vs. 61.5% ingreso en UCI (p=0.006)	Those who receive vitamin D need less oxygen therapy and/or admission to the ICU
Cereceda E et al.[27]	Cases/Controls	Patients with COVID-19: Parkinson's disease (105), caretakers (92), hospitalized (127)	Cholecalciferol ≥800IU/day in 38 individuals	Mortality intrahospital	OR=1.78 (0.64-4.91; p=0.26)	Those who receive vitamin D are more likely to die
Entrenas-Castillo et al.[28]	Clinical trial open pilot, randomized and double blind	Hospitalized by COVID-19 pneumonia (76; 50 treated and 26 untreated)	Calcifediol 64,000 IU/1st week and subsequently 16,000 IU/week until discharge or admission to the ICU	Admission to ICU	2% vs. 50% (p<0.001)	Those who receive vitamin D are admitted less to the ICU, although the risk factors are not balanced between groups. Doses are higher than recommended.

ICU: Intensive Care Unit.

been obtained, although their limitations should be taken into account[23-28]. There are no data on baseline and final 25-hydroxyvitamin D values, although they all assess important outcome variables, such as incidence of disease and mortality.

Some studies in an institutionalized geriatric population that analyze the effect of boluses of cholecalciferol (80,000 IU) prior and/or at the time of infection, report a better evolution of the disease and a decrease in mortality, while in other studies of the same characteristics, this effect is observed in individuals who are treated with periodic boluses of cholecalciferol during the year prior to infection[24,25]. In both cases, the doses used are higher than those recommended. In a study carried out in China with a cohort of asymptomatic patients with COVID-19, the effect of the administration of supplements associating cholecalciferol (1,000 IU), magnesium and vitamin B12 on the evolution of the disease was assessed[26]. Those who received supplements were admitted to the intensive care units (ICU) less and required less oxygen therapy. However, another study, with cholecalciferol, did not confirm these data. The administration of supplements was associated with a tendency to increase mortality, although not statistically significant[27]. However, it is important to mention the methodological limitations of these studies. The only study with calcifediol (25-hydroxyvitamin D) has been carried out in Spain and shows a reduction in the severity of the disease and in mortality. Relatively high doses of calcifediol were used (0.532 mg, followed by 0.266 mg at 3 and 7 days and subsequently weekly until the patient was discharged), without baseline or during vitamin D treatment determinations, which could raise safety concerns[28]. In fact, with the administration of calcifediol (0.266 mg) every two weeks, 25-hydroxyvitamin D concentrations greater than 30 ng/ml are reached in most individuals. Although with this type of dosage, the development of hypercalcemia, around 38% of individuals present concentrations greater than 60 ng/ml[29]. Another study with weekly dosing showed mean concentrations of 93.2±32.4 ng/ml[30].

Although it seems reasonable to use faster and more powerful supplements to achieve sufficient concentrations of vitamin D, it is advisable to carefully consider the dose and frequency of administration.

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At the moment, it is not known what is the optimal vitamin D threshold that we must achieve in the prevention or treatment against COVID-19 to reach the objective, as well as the doses that should be used. In a study carried out in China with a small sample size (62 cases and 80 controls), this threshold was set at 16.5 ng/ml[31]. It seems reasonable to achieve levels above 20 ng/ml and preferably above 30 ng/ml.

In conclusion, we can say that the evidence to indicate the use of vitamin D in preventing and/or treating COVID-19 is scarce and with numerous limitations, with insufficient clinical information to recommend one or another metabolite.

What is the risk/benefit ratio of its administration?

Pending the publication of clinical trials that confirm or not its usefulness, the risk/benefit ratio could be favorable to the use of vitamin D in compassionate use (off-label) in the prevention and treatment of COVID-19 in patients with risk, in which it might be reasonable to prevent or treat deficiency, given the known beneficial effect on immunity and respiratory infections.

What is the risk/benefit ratio of its administration?

In the SARS-COV-2 infection, various treatments have been used, with a certain biological basis for their possible usefulness, but without supporting evidence. In addition to the effective measures implemented (use of masks, distancing, reduced social contact), it is necessary to find some pharmacological measure that reduces the incidence of infection (prevention) or improves its prognosis (therapy). As mentioned before, a higher frequency of hypovitaminosis D has been found in patients with COVID-19.

This finding has been previously observed also in chronic diseases and in some acute diseases, such as respiratory infections. However, the causality of this association has not been clearly established. In the case of SARS-COV-2 infection, there is a biological plausibility for the possible beneficial effect of vitamin D. However, the available evidence is scarce, although, with its limitations, it tends to show a favorable effect.

When using a drug as "compassionate use" without sufficient clinical evidence, the risk/benefit ratio must be assessed. Thus, the first aspect to consider is security. Vitamin D supplements are safe, with very few cases of toxicity, hypercalcemia being the most serious manifestation. The administration of 10,000 IU/day of cholecalciferol or 4,000 IU/day of calcifediol is considered safe[32]. A safety threshold for serum levels of 25-hydroxyvitamin D has been established at 80 ng/ml, while clinical manifestations could appear from 100 ng/ml. Lewiecki[33], in a recent editorial, comments that vitamin D in the treatment of COVID-19 is not dangerous and probably harmless. The risk/benefit ratio could be favorable to the use of vitamin D in compassionate use, off-label, in the prevention and treatment of COVID-19, pending the publication of clinical trials that confirm or not its usefulness. However, given the known beneficial effect on immunity and respiratory infections, it seems reasonable to prevent or treat the deficit in patients at risk.

Conflict of interests: José Luis Pérez Castrillón has participated in clinical trials, work groups, training presentations and attendance at medical conferences funded by FAES, Italfármaco and Gebro-Pharma. Enrique Casado has received fees for conferences, scientific advice or funding for conferences from Italfármaco, Gebro, FAES and Angelini.
Luis Corral Gudino has no relevant conflicts of interest for this article. Carlos Gómez Alonso has participated in clinical trials, work groups, training presentations and attendance at medical conferences funded by FAES, Italfarmaco and Gebro-Pharma. Pilar Peris has collaborated as a speaker with Amgen, Lilly, Kyowa Kirin, UCB and Angelini Pharma. Jose A. Riancho has no relevant conflicts of interest for this article.

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