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Is the bone tissue of the femoral neck demineralisedin patients with hip fracture?

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ABSTRACT

The aim of this study is to establish the falsifiability of the "osteoporotic hypothesis" for hip fracture, according to which the bone density and mineral composition of bone tissue in patients with hip fracture is poorer than when no such fracture is present, and that this circumstance is relevant to the occurrence of a fracture. The study population consisted of forty patients treated with arthroplasty. Twenty patients with femoral neck fracture and another twenty with hip osteoarthritis received the same diagnostic protocol and the same antibiotic, anaesthetic, surgical and antithrombotic prophylaxis. Levels of calcium (Ca), phosphorus (P) and vitamin D in blood, amongst other values, were determined, and five samples of bone tissue from the proximal femoral metaphysis were obtained and characterised by optical microscopy and microanalytical analysis. No statistically significant differences were observed between the two groups with respect to the trabecular number, area or thickness, or inter-trabecular distance. However, there were differences in the length of the trabeculae, which was greater in the patients with hip osteoarthritis ($p = 0.002$), but not when the groups were compared by gender. When compared by age, a greater inter-trabecular distance was observed in the patients aged over 75 years ($p = 0.036$) but there were no differences in the remaining parameters. Serum levels of Ca ($p = 0.03$), P ($p < 0.01$) and vitamin D ($p < 0.01$) were lower in the fracture group. In the quantitative microanalytical analysis, no significant differences were observed in bone levels of Ca or P or in the Ca/P index, nor was there any correlation between serum and levels of bone Ca or P (Ca-0.197:$p = 0.314$;P-0.274:$p = 0.158$).Multiple linear regression revealed no correlation between the diagnoses, vitamin D and bone levels of Ca or P. Despite the reduced serum levels of Ca and P in the patients with hip fracture, no correlation was observed with bone levels of Ca and P,which were similar in both groups. There were differences in the organic bone structure, in terms of length and inter-trabecular distance. For patients with osteoporosis, treatment should be aimed at increasing the synthesis of bone trabeculae to reinforce their structure. Nevertheless, no such treatment can prevent falls, and therefore no reduction in hip fractures amongst this population can be assured.

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Introduction

Hip fracture in old age is an important public health problem, not only because of the high prevalence and morbidity-mortality it provokes, but also because of the direct costs of health services required and other indirect costs generated. In Spain, the incidence of hip fracture increases exponentially from 107 cases per year per 100,000 persons aged 65–69 years to 3992 cases per year per 100,000 persons aged over 94 years [1].

Osteoporosis is often claimed to be a necessary cause of such fractures. This condition is defined as a bone mineral density (BMD) with a T-score of less than −2.5, measured by the absorption of dual emission X-rays. About 30% of women and 12% of men present osteoporosis at some time in their lives [2]. Other variables have also been recognised [3,4], but current strategies for fracture prevention are based on screening for osteoporosis by bone densitometry, followed by appropriate treatment [5–10]. However, the validity of bone densitometry as a means of defining osteoporosis as a disease has been seriously questioned [11,12], with critics arguing that BMD provides little information on fracture risk, and that the Black scale (or Fracture Index) is more predictive [9].

In fact, numerous studies have concluded that the main risk factor for hip fracture in the elderly is the accidental fall, not osteoporosis [13–15]. Hence, providing treatment for osteoporosis not only does not decrease the annual incidence of hip fracture but it creates an addi-

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tional cost, whilst achieving debatable therapeutic effects [16]. Hip fracture is undoubtedly preceded by trauma, and there would be no hip fracture without trauma (except in the infrequent case of a lytic lesion). Systematic reviews and meta-analyses of randomised trials show that at least 15% of falls by elderly patients can be avoided, and some studies place this figure at 50% [14,17]. Therefore, preventing falls decreases the risk of fractures [18].

The aim of this study is to establish the falsifiability [19] of the "osteoporotic hypothesis" according to which the bone density and mineral composition of the bone tissue in persons with hip fracture is lower than in those with no fracture, which facilitates the appearance of the condition.

Patients and methods

The study population consisted of forty consecutive patients admitted to hospital for total hip arthroplasty. Twenty of these patients were diagnosed with fracture of the femoral neck and the other twenty, with hip osteoarthritis. All patients received, sequentially, the same diagnostic protocol and antibiotic, anaesthetic, surgical and antithrombotic prophylaxis.

The selection criteria applied ensured that both groups presented comparable age, gender and anaesthetic risk, with no significant differences (Table 1). Patients with a disease or receiving treatment that directly interfered with bone metabolism, or who presented a pathological fracture or hip osteoarthritis secondary to non-mechanical disease, or who had a history of ipsilateral hip fracture or severe psychiatric disorder, or who were concurrently admitted to hospital or whose life expectancy was less than six months were excluded from the study.

All the patients included in the study gave oral and written informed consent for the diagnostic and therapeutic procedures performed, and agreed to take part in this research project, which was authorised by the hospital's ethics committee and research commission (Act number 43, 09 July 2010).

On admission, a blood sample was taken from each patient (two tubes for blood count, two for biochemical analysis and one for coagulation), to study calcium (Ca), phosphorus (P) and vitamin D in serum, amongst other parameters. In addition, during the surgical procedure five samples of bone tissue were taken from the proximal femoral metaphyseal area for subsequent optical and electron microscopy study and for qualitative and quantitative microanalytical analysis.

Table 1
Independent variables.Statistical significance was assumed at $p < 0.05$.

	Hip Osteoarthritis		Fracture		p
	N (20)	%	N (20)	%	
Gender					
Male	6	30	4	20	0.715
Female	14	70	16	80	
ASA					
1 × 2	14	70	8	40	0.112
3 × 4	6	30	12	60	
Age					
Median (IQR)	*74*	*8.5*	*76*	*11.25*	0.383
BMI					
Median (IQR)	*29.4*	*4.9*	*26.2*	*5.8*	**0.047**

Histological study

The samples were fixed in 4% buffered formaldehyde for 24 h at room temperature and then demineralised by immersion with continuous gentle agitation in EDTA, for seven days, until the total disappearance of the hydroxyapatite. After washing, the samples were inserted in paraffin before dehydration in a decreasing solution of ethanol and xylol, with cuts at 5 μ thickness and conventional staining with haematoxylin and aqueous eosin. Three different images of each sample were taken at 4x magnification, and processed using Image J 1.51 w software (Wayne Rasband. National Institutes of Health, USA http: //imageJ.nih.gov.ij Java 1.8.0_181; 32bit) connected to a Nikon Eclipse i90 optical microscope.

Electron microscopy study

The samples were cryofixed with liquid nitrogen [20,21] and vacuum freeze-dried (Freeze-drier Polaron E 5300), by sublimation from the solid phase, starting at −100 °C and increasing progressively, over 24 h, to 25 °C, at which point they were mounted in a graphite sample holder. The samples were then carbon coated for 30 s (Sputtering Polaron E-5000), using argon (P = 0.1 Torr). Finally, they were observed by high-vacuum scanning electron microscopy (Philips XL-30).

Qualitative microanalytic study

This analysis was carried out using a scanning electron microscope (Philips XL-30) equipped with an energy-dispersive X-ray detector. The microscope and detector parameters were maintained at constant values of 15 Kv, 40000x magnification, 35° surface angle, 52° perception angle, 1200 counts per second and an accumulated count time of 100 s. With these parameters, a series of qualitative spectra were obtained for each sample. In these spectra, the Ca and P levels were selected in their K-alpha orbitals, and the counts per second (CPS), the background or non-characteristic radiation and the peak/background index (P/B) were determined.

Quantitative microanalytic study

The concentrations of Ca and P in the weight fraction were quantified by a modification of Hall's method [22], namely the P/B method proposed by Staham and Pawley [23] and by Small et al. [24], which takes into account the background or non-characteristic radiation in the same energy range as the characteristic peak and continues within the irradiated volume. Amongst the advantages of this method are its independence of variations in the intensity of the beam current and of the effects on the surface of the specimen, and its applicability to the quantitative analysis of rough surfaces in scanning electron microscopy. To do this, the following standard Ca and P salts were used: $Ca_2P_2O_7$, $C_6H_{11}O_7.1/2Ca$, $CaHPO_4$, $Ca\ (H_2PO_4)_2$, $PO_4HCa.2H_2O$, $C_{12}H_{21}O_{12}.1/2Ca$, $CaSO_4.2H_2O$, $CaH_4O_8P_2.H_2O$, $Ca_3O_8P_2$, $P_3O_9Na_3$, PO_4HK_2, processed under identical methodological guidelines to those used for the specimens [25–28]. Ten quantitative determinations were taken for each of the samples, with 200 quantitative microanalysis determinations per group. For each sample, the median value of the ten determinations was obtained. The median value for the whole study group was also determined.

Statistical analysis

The data obtained were entered into an Excel spreadsheet (Microsoft, Redmond, USA) and IBM SPSS V15 statistical software was then used to perform the descriptive analysis, with position measure-

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ments (median and interquartile range) for the quantitative variables and frequency distributions for the qualitative ones. For the qualitative variables, the differences between diagnoses were evaluated by the chi-square test (or by Fisher's test when fewer than five frequencies were expected). The Mann-Whitney U test was used for the quantitative variables. A multiple linear regression model was constructed to evaluate the influence of the diagnosis and of vitamin D with respect to average Ca and P levels in bone, and correlations between the variables were evaluated by Spearman's Rho test. Statistical significance was assumed at $p < 0.05$.

Results

Independent variables

Both groups presented comparable age, gender and anaesthetic risk, with no significant differences. There are significant differences in BMI, $p = 0.047$ (Table 1).

Optical microscopy study

No statistically significant differences were observed between the two diagnostic groups in terms of trabecular number, area or thickness,or intertrabecular distance. On the other hand, differences were found in the average length of the trabeculae, which was greater in the group of patients with hip osteoarthritis (Figs. 1–3), although not when the groups were compared by gender. In the comparison by age, a greater intertrabecular distance was observed in patients aged over 75 years (median age) (Figs. 4–5). No other differences were observed for this age group, (Table 2).

Serum levels of Ca, Pand vitamin D

Statistically significant differences were found between the two diagnostic groups in the serum levels of Ca and P. Blood levels of vitamin D were low in both groups,but significantly lower in the hip fracture group, (Table 3, Fig. 6).

Qualitative microanalytical analysis

Before performing the quantitative microanalytical analysis of the bone samples, the presence of Ca and P in the K-alpha orbitals was qualitatively determined, (Figs. 7–8), in terms of counts per second (CPS), non-characteristic or background radiation (BKGD) and peak/background index (P/B).

Quantitative microanalytical analysisss

No statistically significant differences were observed between the groups in the levels of Ca or P or in the Ca/P index, (Table 4).

Ca and p correlation in serum and bone

No correlation was observed between Ca or P in serum and the levels obtained by the microanalytic study in bone, according to Spearman's Rho test.The correlation coefficients obtained were −0.197 ($p = 0.314$) for Ca and −0.274, ($p = 0.158$) for P.

Multivariable study

The multiple linear regression model revealed no correlation between the diagnostic groups with respect tolevels of vitamin D in serum and of Ca and P in bone.

Discussion

Hip fracture management in the elderly continues to be a challenge [29–32]. This injury is usually considered to be due to bone fragility, and correlated with osteoporosis. However, numerous studies have shown that falls are the main risk factor for fractures in the elderly [13–15]. All older people have osteoporosis, but only some will suffer a fall; and of these, fewer than half will be injured as a result [16].

Research evidence shows that exercise and gait training decrease the risk of fracture [33] and the findings of some randomised trials

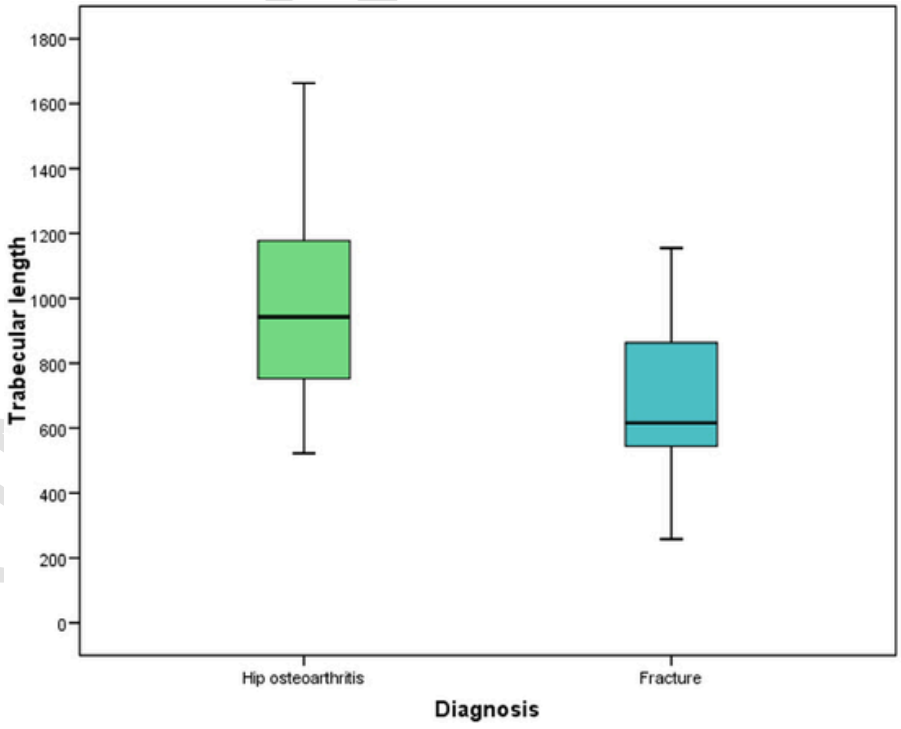

Fig. 1. Trabecular length by diagnostic group, assessed by optical microscopy at 4x, is greater in patients with hip osteoarthritis than in those with hip fracture.

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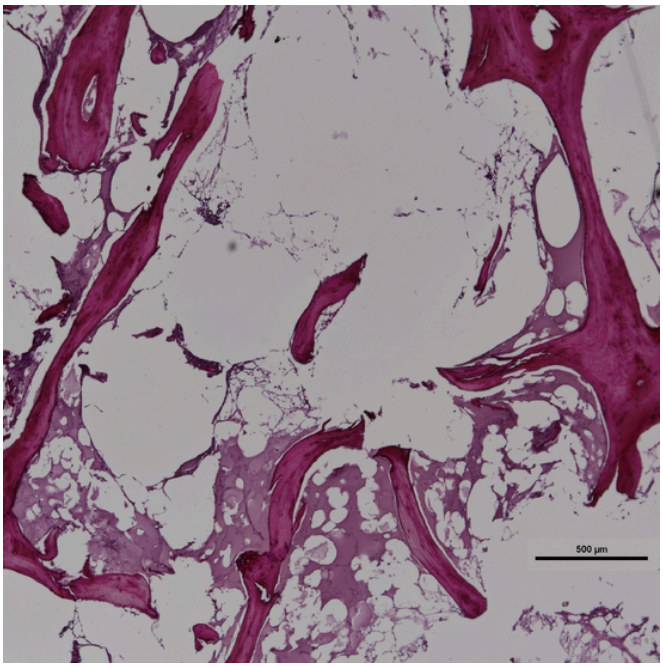

Fig. 2. Optical microscopy study of bone sample with haematoxylin and eosin staining, in a patient with hip osteoarthritis (4x magnification).

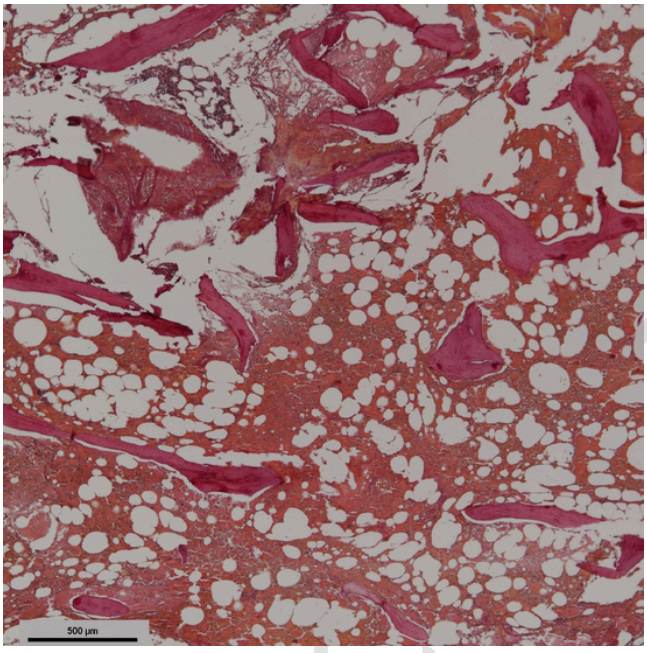

Fig. 3. Optical microscopy study of bone sample with haematoxylin and eosin staining, in a patient with hip fracture (4x magnification).

suggest that more specific actions should be taken to prevent falls, such as preferential cataract surgery or the use of cardiac pacemakers (when indicated), together with the use of anti-slip and gait-stabilising devices [13,34,35].Although these research findings on the prevention of falls have insufficient statistical power for fracture to be defined as the main outcome variable, several randomised studies have concluded that preventing or reducing the number of falls in the elderly does reduce the number of fractures, sometimes by up to 50% [34,36,37]. A meta-analysis of studies of interventions to prevent falls showed that the relative risk of injury-provoking falls could be reduced in the same proportion as that of non-injury falls (35%) [33]. Studies have also shown that related treatments such as cataract surgery are also associated with reduced numbers of falls and hence a decreased incidence of fractures and related problems [34,38].

This view of the problem suggestst hat new lines of research might profitably be pursued. These could be classified into two basic profiles: one of an epidemiological nature on the causal relationship between falling and the individual's pathological history, and the other to study possible differences in bone tissue, with particular regard to the protein structure that is mineralised, in terms of the organic and mineral phases. In both cases, to verify falsifiability [19] it is necessary to study the correlation between the characteristics of bone tissue or falls and the appearance of fractures.

In our study, the Body Mass Index (BMI) was significantly lower in the group of patients with hip fracture (Table 1). Cohort studies and meta-analyses have shown that low BMI is associated with a greater risk of hip fracture, independently of bone mineral density (although the exact mechanisms that explain this relationship are unclear). Nevertheless,even when the BMI is within normal limits, it is associated with bone loss and, therefore, with a greater probability of fracture [39]. On the other hand, it has also been suggested that adipose tissue around the hip area may absorb part of the impact of a fall and thus protect against fracture, and it has been observed that the protective effect of high BMI is greater than that of low and moderate BMI. However, the presence of high BMI (> 25 kg/m^2)is not recommended, due to its association with other morbidities [40,41]. The median BMI for both diagnostic groups in our study was above this limit of 25 kg/m^2. In the patients diagnosed with hip osteoarthritis, the median value was 29 kg/m^2, with an interquartile range (IQR) of 4.9, and in the fracture group it was 26.2 kg/m^2, with an IQR of 5.8 (Table 1). However, the BMI may also be indicative of malnutrition, which would facilitate sarcopenia and a propensity to suffer falls [42].

Crystalline hydroxyapatite is the main mineral component of bone, constituting about a quarter of the volume and half of the mass of normal adult bone. The Ca and P (inorganic phosphate) components of these crystals are derived from blood plasma and, in turn, from nutritional sources. Amorphous Ca phosphate matures through several intermediate stages to form hydroxyapatite, and the metabolites of vitamin D are important mediators of Ca regulation.Vitamin D deficiency will result in the depletion of bone minerals [43].However, in our study, although the levels of Ca, P and vitamin D in blood were reduced, their concentration in the bone was not affected. There is little evidence of a relationship between bone density and Ca intake, but Ca suplements are associated with the appearance of adverse effects such as gastrointestinal problems, kidney stones and even cardiovascular problems [40]. Therefore, treating osteoporosis by Ca and vitamin D supplementation does not seem appropriate if there is no vitamin or Ca deficiency [44–47].

Studies of the nanostructure, composition and microarchitecture of the superolateral area of the femoral neck in elderly patients with hip fracture, compared to healthy controls, have shown that the mineral crystals on the external cortical bone surfaces of the fracture group are larger, with a greater mineral content, and present a more homogeneous mineralisation profile.In the study of Milovanovic et al , however, samples from the patients with hip fracture revealed cortical porosity values that were almost 35% higher than in the control group [48]. In the osteoporosis and osteoarthrosis groups, the Ca-P ratio did not vary between the lacunae of hypermineralised osteocytes and the bone matrix. Although the role of hypermineralised osteocyte lacunae in bone remodelling and the biomechanical properties of bone require further investigation, these findings are very interesting, suggesting that hypermineralisation is associated with susceptibility to femoral neck fracture [49].

In our study, in both groups bone samples were taken from the proximal metaphysis of the femur. This contrasts with other studies,

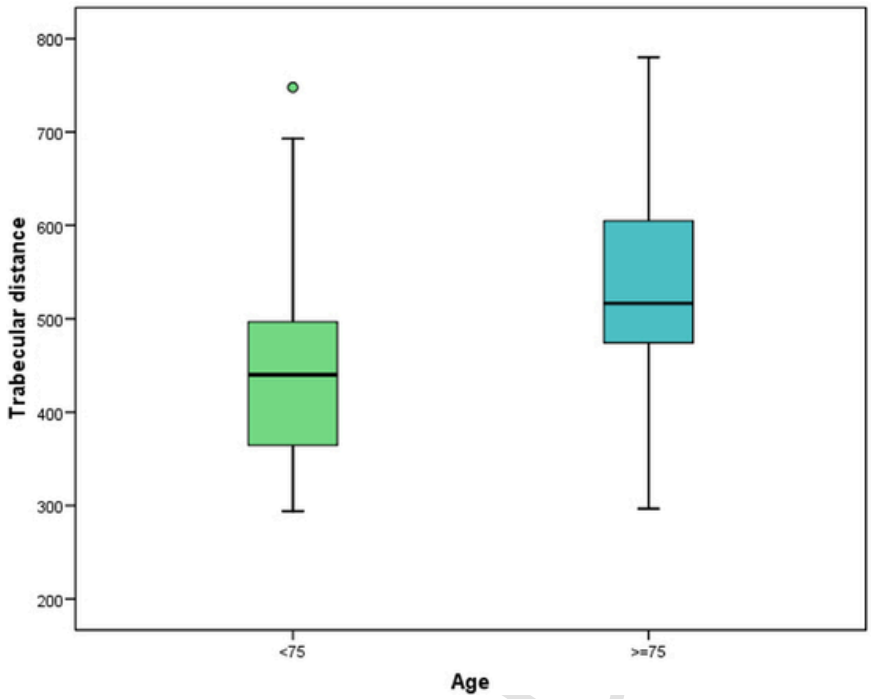

Fig. 4. The intertrabecular distance, assessed by optical microscopy, is greater in patients aged over 75 years (4x magnification).

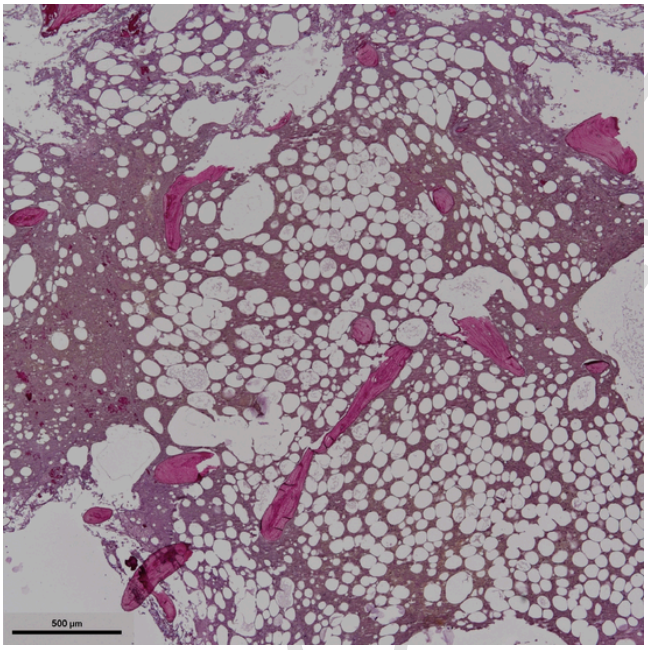

Fig. 5. Optical microscopy study of a bone sample with haematoxylin and eosin staining, in an 83-year-old patient with hip fracture, revealing considerable distance between the trabeculae (4x magnification).

which did so from the femoral head, a method that may provoke selection bias,due to the greater bone density of the femoral head in patients with hip osteoarthritis. The results for all samples obtained from both groups were calculated in terms of the weight ratio of Ca and P [26].The differences were not statistically significant for Ca, P or the Ca/P index. These results show that the mineral composition determined by quantitative microanalysis of cancellous bone obtained from patients with osteoarthrosis of the hip is similar to that of the bone from patients with hip fracture.This finding, associated with that of altered serum concentrations of Ca and P (serum/bone levels: correlation coefficient −0.197 (p = 0.314) for Ca and −0.274 (p = 0.158) for P), refutes the idea that Ca intake should be increased or medications administered to increase mineralisation in patients with osteoporosis, in order to prevent hip fracture. However,it is consistent with the recommendation that Ca and P fractions in bone mineral density should be determined, in order to improve the assessment of fracture risk and to facilitate the provision of more specific treatment [50].

In relation to the organic phase of bone samples, the number of trabeculae and their connectivity are very significant to the biomechanical behaviour of cancellous bone. A structure with a greater number and thickness of trabeculae and greater inter-trabecular connectivity will be more resistant [51]. In our study, no differences were observed between the diagnostic groups with respect to the number of trabeculae, their thickness or the inter-trabecular distance, although the value for thickness was very close to statistical significance (p = 0.051). However, there were inter-group differences in trabecular length, which was greater in the patients with hip osteoarthritis. By gender, no significant differences were observed between the groups for any variable. However, when the groups were divided according to the median age (75 years), statistically significant differences were observed in inter-trabecular distance (Table 2). These findings could account for the epidemiology of hip fracture in older patients, in terms of greater distance between the trabeculae, the presence of shorter, thinner trabeculae, and the existence of more severe fracture patterns.

Despite the significant decrease in levels of Ca and P in serum in patients with hip fracture, there was no correlation with levels in bone, which were similar in both groups. However, there were differences in the organic bone structure in terms of trabecular length and thickness, which suggests that new hypotheses might be considered.On the one hand, the therapeutic administration of Ca and P to prevent fractures, and that of drugs to facilitate intestinal absorption, appear to be irrelevant whenthere is no deficiency of these minerals in bone, as discussed above. However, as they are involved in muscle metabolism, it could be hypothesised that low levels of Ca and P in serum, with normal bone levels, might be associated with fractures from falls, as a result of altered muscle physiology and senile sarcopenia, apart from the influence of other age-related disabilities.The appearance of malnutrition in pa-

Table 2
Optical microscopy by diagnosis, sex and age.Statistical significance was assumed at $p < 0.05$.

Optical Microscopy			Trabecular area	Trabecular thickness µm	Trabecular lenght µm	Intertrabecular Distance µm	Trabecular number 4x
Total	Hip Osteoarthritis	Median	0.13	156.10	942.78	476.25	5.67
		IQR	0.08	70.76	448.94	284.95	1.88
	Fracture	Median	0.09	115.56	616.61	479.07	6.00
		IQR	0.07	28.64	322.07	168.15	2.25
		p	*0.117*	*0.051*	***0.002***	*0.957*	*0.498*
Gender	Male	Median	0.10	134.37	906.62	527.93	6.00
		IQR	0.06	59.53	504.54	447.13	2.50
	Female	Median	0.09	119.40	791.86	475.29	5.67
		IQR	0.07	50.39	347.88	132.24	1.75
		p	*0.574*	*0.685*	*0.975*	*0.708*	*0.552*
Age	<75	Median	0.09	118.36	862.51	440.11	6.33
		IQR	0.07	59.42	537.07	158.11	2.33
	>75	Median	0.09	128.40	790.96	516.48	5.67
		IQR	0.06	47.51	412.47	161.72	2.00
		p	*0.524*	*0.507*	*0.297*	***0.036***	*0.162*

Table 3
Serum levels of calcium, phosphorus and vitamin D.

	Hip Osteoarthritis		Fracture		p
	Median	IQR	Median	IQR	
Calcium(mg/dl)	*9.15*	*0.45*	*8.65*	*0.60*	**0.03**
Phosphorus(mg/dl)	*3.30*	*0.45*	*2.85*	*0.77*	<**0.001**
Vitamin D (ng/ml)	*23.90*	*14.71*	*8.70*	*9.30*	<**0.001**

tients with hip fracture has been amply demonstrated [52], and it is also a risk factor for sarcopenia [42,53]. Treatment for osteoporosis should be aimed at increasing the synthesis of bone trabeculae to reinforce their structure, and this approach has implications for nutrition prior to the appearance of fracture. In our study, the BMI was lower in the patients with hip fracture, which can be an indicator of malnutrition [54]. Nevertheless, none of these treatments actually prevent falls, and so the reduction of hip fractures in this population cannot be assured by such methods alone.

Declaration of Competing Interests

None of the authors has received any economic consideration from third parties. There is no conflict of interest.

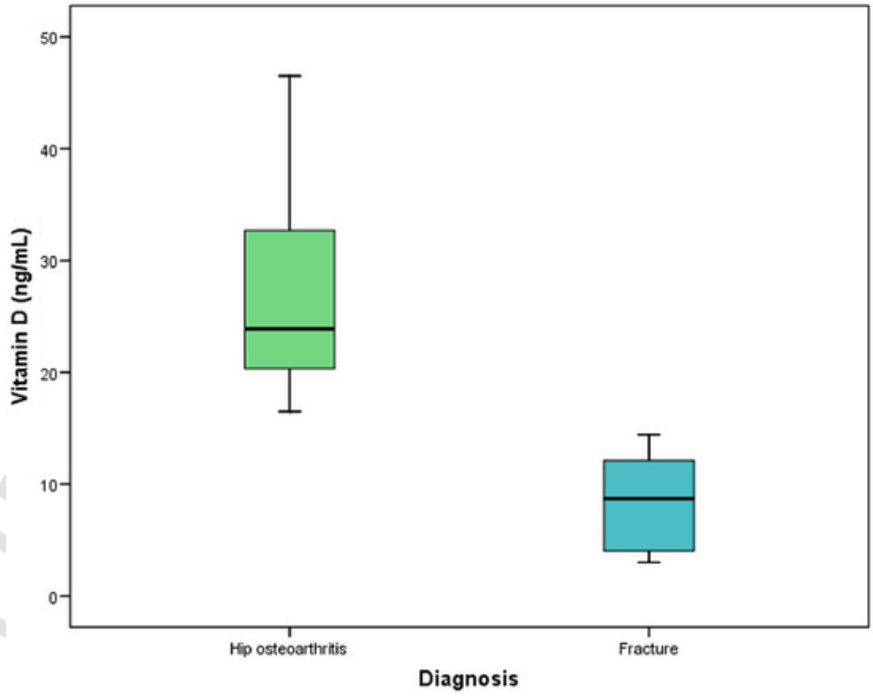

Fig. 6. Serum vitamin D levels are reduced in both groups, but especially in the patients with hip fracture.

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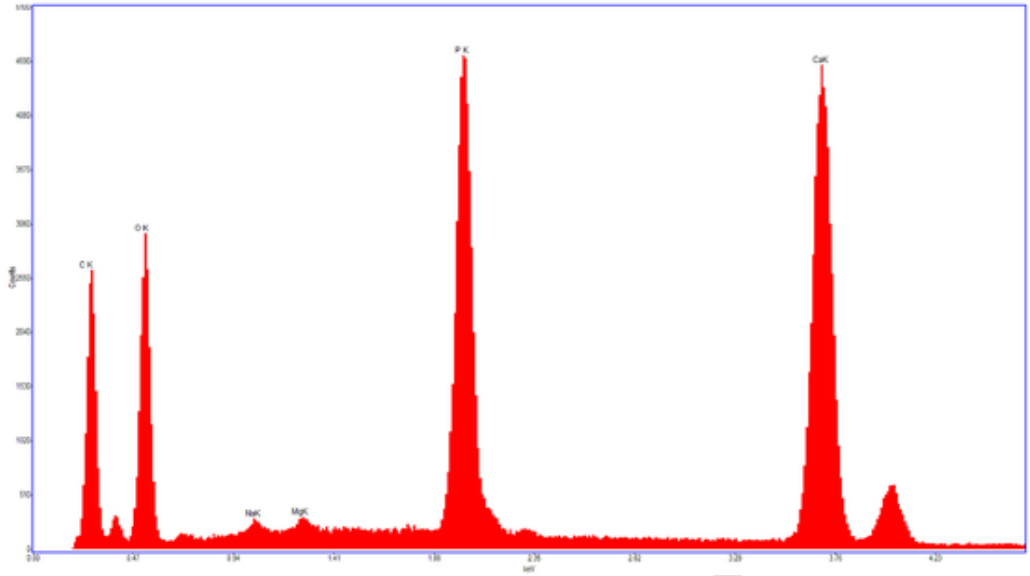

Fig. 7. Qualitative microanalytical spectrum of a patient with hip osteoarthritis. The existence of Ca and P is apparent, together with other minerals in the K-alpha orbital.

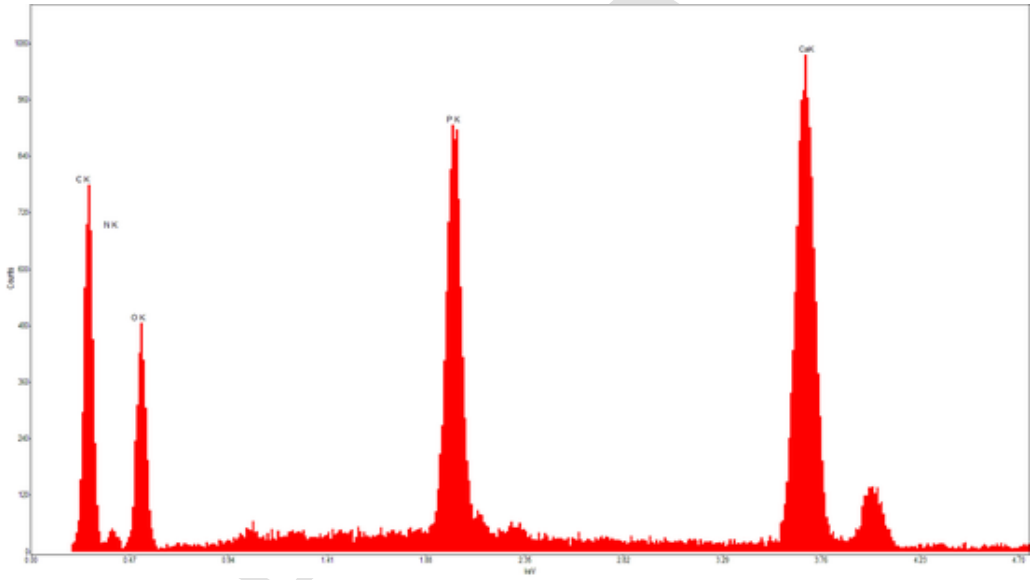

Fig. 8. Qualitative microanalytical spectrum of a patient with hip fracture. The existence of Ca and P is apparent, together with other minerals in the K-alpha orbital.

Table 4
Bone levels of calcium and phosphorus, and the calcium/phosphorus index.

	Hip Osteoarthritis		Fracture		p
	Median	IQR	Median	IQR	
Bone Calcium	*28.38*	7.29	*28.19*	*7.17*	0.75
Bone Phosphorus	*11.36*	*2.17*	*11.40*	*1.37*	0.77
Calcium/Phosphorus index	*2.29*	*0.55*	*2.41*	*0.45*	0.42

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