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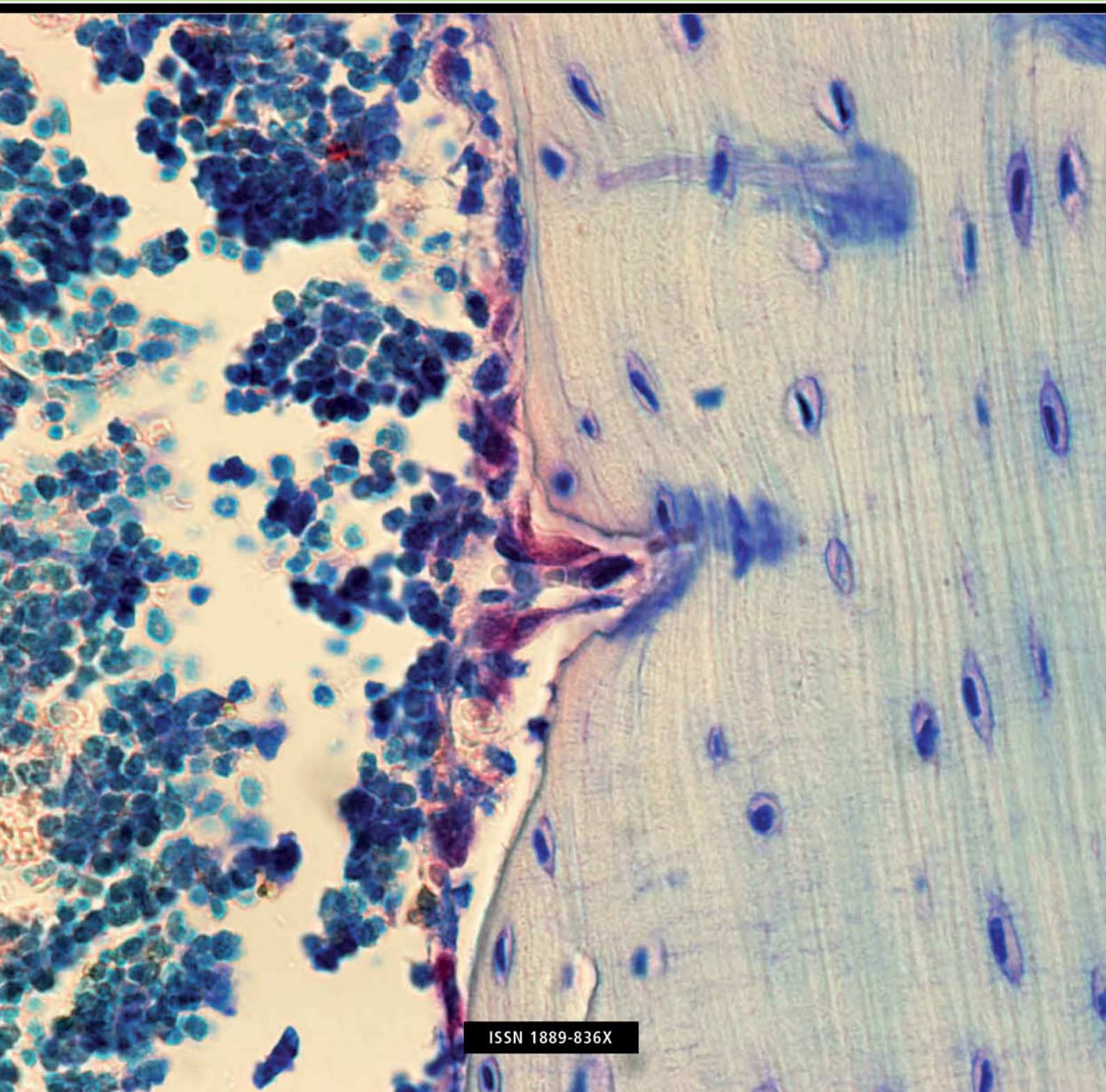

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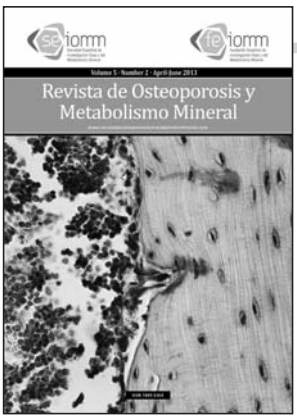

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Inadequate levels of D: not a D-elicious perspective

Quesada Gómez JM, Navarro Valverde C

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In the last few decades clinical research in large population studies has revealed the high prevalence of insufficient levels of vitamin D across the globe[1], which, combined with its effects on bone, the muscular-skeletal system, innate and acquired immunity, the cardiovascular system, and the development and function of cells, makes this a first order problem in public health. In fact, low levels of vitamin D are associated significantly with all the causes of morbimortality[2].

The description in this number of the Review of Osteoporosis and Mineral Metabolism of inadequate levels of vitamin D in patients with spinal injury in Las Palmas de Gran Canaria, a city in Spain which, due to its climate, is a paradigm for the ease of obtaining vitamin D_3 by cutaneous synthesis throughout the year, again challenges us[3]. Due to its high prevalence, its ease of detection, its associated adverse consequences, and its simple, cheap and efficacious means of treatment, vitamin D insufficiency should be an urgent and immediate priority for health services in general and for every medic, irrespective of their specialism.

Blood levels of 25 hydroxyvitamin D are considered to be a marker for the status of vitamin D in the body, including endogenous synthesis from to exposure to the sun, dietary ingestion of foods with or without supplements or drug treatments[4].

However, blood levels of 25 hydroxyvitamin D are not strictly regulated, no method for their quantification is perfect, with a great variability between laboratories, even the most properly checked, which, therefore, makes the definition of normality difficult[5]. In fact those methods which do not use high pressure separation chromatography do not distinguish between the metabolites of vitamin D2 or vitamin D_3[6]. Although in Spain this is not a problem since vitamin D_2 is not used in normal clinical practice, many metabolites of vitamin D are quantified as 25 hydroxyvitamin D, 24,25 dihydroxyvitamin D_3, its epimer C-3, or sulphated forms, etc., and this problem persists even when using high pressure liquid chromatography separation and mass measurement[7].

Nevertheless, while they need to improve substantially, the methods available in our normal practice of treatment or research are adequate enough and should be used more in our clinical practice for the diagnosis and follow up of treatment.

So, in the face of the variability of the different laboratories and different testing methods, the controversy regarding cut off points for normal blood levels of 25 hydroxyvitamin D proposed by different scientific societies, above 20 ng/ml for the Institute of Medicine[3] and above 30 ng/ml for the International Osteoporosis Foundation (IOF)[8], supported by the recommendation of the Endocrinology Society in the US[9], is a byzantine argument, and only one way of approaching the problem.

In any case, we could agree that the objective should be to achieve blood levels higher than 20 ng/ml as a bare minimum, and preferably higher than 30 ng/ml. So, if our patients have levels of 25 hydroxyvitamin D above 30 ng/ml we will be in agreement with existing recommendations .

Blood levels higher than 30 ng/ml will foster proper bone health and an effective response to antiresorptive treatments for osteoporosis[10,11], in addition to its more than likely beneficial impact on practically all the body's organs and systems[2,12].

Defining the maximum values is more critical. Although for some time it has been proposed that high values of vitamin D, except above toxic values, would not be damaging, there is currently

an open debate as to whether high levels of 25 hydroxyvitamin D may be associated with risk of cardiovascular death, or death by other causes[13]. Therefore, some authors have proposed a recommended cut off point blood levels of 25 hydroxyvitamin D of 60 to 70 ng/ml quantified by the usual measurement methods, which are values present in the Summer in agricultural workers, fishermen, lifeguards at the beach or swimming pool etc., who have a high exposure to ultraviolet rays and an intense epidermic production of vitamin D_3, but who never exhibit toxicity[3].

In the treatment of vitamin D insufficiency/deficiency, after the optimisation of 25 hydroxyvitamin D levels within the range of 30 to 70 ng/ml, we should not forget its maintenance once this range is reached.

To achieve this objective, in Spain we may use vitamin D_3 or 25 hydroxyvitamin D_3 (calcifediol), which means that we must remember that contra to what we have come to believe for many years, these metabolites are not equipotent, but that the latter is approximately three times more powerful than the former[14,15].

Finally, we would like to stress that, regarding the already recognised high prevalence in Spain of insufficient levels of vitamin D in all sections of the population studied[16,17], which is always greatest in patients with conventional risk factors for having low blood levels of vitamin D, the increase in obesity and poverty[18] will presumably make the current critical situation worse in the coming years.

Unfortunately, current public health policies do not auger well for effective change in the face of this serious problem, which is why we must end our comments with a quote from Robert Heaney, ending one of his interventions at the VI Symposium of SEIOMM held in Granada in October 1997 on the problem of vitamin D deficiency: "....we have the evidence; but when will we see the action.".

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Prevalence of hypovitaminosis D and secondary hyperparathyroidism in the Spinal Cord Injury Unit in Gran Canaria. Preliminary study

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Summary
Background: vitamin D deficiency is very common, and has been demonstrated in multiple studies in both the general population and in patients with different pathologies. However, it has been little studied in patients affected by spinal injury.
Objective: to study the prevalence of hypovitaminosis D and the possible development of secondary hyperparathyroidism in a population of patients with spinal injury.
Material and method: transverse descriptive study carried out in 104 patients affected by spinal injury. A clinical history was taken, a detailed physical examination carried out and a blood sample while fasting taken, with the least possible compression, from all patients. The analytical parameters were analysed using automated techniques and the determination of 25-hydroxyvitamin D (25HCC) and parathyroid hormone (PTH) was performed using electroimmunochemiluminescence (ECLIA).
Results: the global mean value of 25-hydroxyvitamin D was 20.1 ± 11.6 n/ml. 84.6% of the patients had blood values of 25-hydroxyvitamin D lower than 30 ng/ml and 62% of all patients showed values lower than 20 ng/ml. The prevalence of vitamin D deficiency was similar in men and women. However, although we found an inverse correlation between levels of PTH and hydroxyvitamin D, only 5.8% of patients ended up developing secondary hyperparathyroidism.
Conclusions: there is a high prevalence of hypovitaminosis D in patients with spinal injury. It is advisable, therefore, to include a study of this metabolite in the care protocol of these patients to correct these deficiencies as and when they are found.

Key words: *vitamin D, parathormone, spinal injury.*

Introduction

Vitamin D plays an important role in bone mineral metabolism since it is involved in the regulation of levels of calcium and phosphorus, and its deficiency may be an epipathogenic factor in osteoporosis. However, in the last few years there has been clear evidence which gives the action of vitamin D highly important effects outside the bone, which fundamentally alters musculo-skeletal function. Recent studies have reported that vitamin D acts on the immune system, prevents pathologies such as arteriosclerosis[1], arterial hypertension[2], resistance to insulin[3] and hyperglycemia[4], in addition to being related to the prevention of various types of cancer[5-7].

Blood levels of vitamin D are a significant risk factor in the diminution of bone mass and in the increased risk of fractures in these patients. Some studies have been published which show deficient levels of vitamin D in patients with spinal injury, the prevalence of this deficit being estimated at between 30 and 32%[8,9].

The measure of blood levels of 25-hydroxyvitamin D (25HCC) is the universally accepted form of indicator for vitamin D reserves[10]. There is not a unanimous consensus regarding the blood levels of 25-hydroxyvitamin D sufficient to ensure bone health in the general population, and even less in patients with spinal injury. However, it tends to be accepted that ideally these levels should be, as a minimum, 30 ng/ml[11].

Patients with spinal injury usually have osteoporosis secondary to immobilisation and fractures, above all in the lower limbs. The additional role which vitamin D deficiency may have in its etiopathology has not been determined. Therefore, we carried out this study in a population of patients with controlled spinal injury in the Spinal Injury Unit of the Island University Hospital Complex for Mothers and Children in Gran Canaria.

Patients and methods

This is a transverse observational study carried out in 104 patients who were admitted to the Spinal Injury Unit of the Island University Hospital and who were seen either at a first visit or at a review, during the year 2012. A required criterion for inclusion in the study was the existence of an irreversible spinal injury. The patients were informed of the objectives of the study, and had previously signed their informed consent to participate. The study was approved by the Committee for Ethics and Clinical Trials of the Island University Hospital.

In all cases we applied the clinical protocol for the study and follow up of patients with spinal injury, which included clinical history, physical examination, biochemical parameters (haemoglobin, glucose, urea, creatinine, calcium, phosphorus, total proteins, lipid profile, tartrate-resistant acid phosphatase (TRAP), PTH, 25-hydroxyvitamin D, beta-crosslaps, osteocalcin and amino-terminal peptide of collagen type 1 - P1PT).

Blood was taken in fasting, with the least possible compression and the general biochemical parameters (haemoglobin, glucose, urea, creatinine, calcium, phosphorus, total proteins, lipid profile) were measured with an autoanalyser; the TRAP by spectrophotometry; and the PTH, 25HCC, beta-crosslaps, osteocalcin and P1NP by electroimmunochemiluminescence (EIQL). The period of collecting the samples extended from March to May 2012.

Normal levels of vitamin D were considered to be where blood values of 25-HCC above 30 ng/ml; insufficiency, those between 20-30 ng/ml; and deficiency, figures below 20 ng/ml, in accordance with the position document of the International Osteoporosis Foundation (IOF)[12]. With respect to PTH, normal values are considered to be <88 ng/ml, as set by the laboratory, and levels higher than this are indicative of secondary hyperparathyroidism.

A descriptive statistical analysis of the study's baseline data was carried out. For this, we calculated the absolute and relative frequencies in the case of the qualitative variables. The quantitative variables were summarised by means of mean ± standard deviation or percentiles with average interquartile range, respectively, depending on whether or not there is a normal distribution after being subject to the Kolmogorov-Smirnov test.

Results

In table 1 we show the characteristics of the population studied. The 104 patients with spinal injury had an average age of 43.4 years, and 74% were men. 86.5% of the patients had had an injury of traumatic origin. 40% were tetraplegic. The average time from the injury occurring to the date of the study was 8 years.

Table 2 shows various analytical value data, such as renal function and lipids (total cholesterol, triglycerides and HDL-cholesterol). The average values of these parameters were within normal values.

In table 3 we show the analytical data related with bone metabolism. The median values of 25-HCC were globally 20.1 ± 11.6 ng/ml, being 20.1 ± 11 ng/ml in men and 19.9 ± 13.5 ng/ml in women (p=0.919). According to established parameters for normality, 84.6% of those with spinal injury had blood values of 25-hydroxyvitamin D lower than 30 ng/ml, and 62.5% had values lower than 20 ng/ml. The prevalence of vitamin D deficiency was similar in both sexes (Table 4).

The analysis as a function of age, with the limit established at 50 years of age, showed that the patients younger than 50 had a higher percentage of vitamin D deficit (66.6%) than those over 50 years of age (53.1%). However, among those over 50 there was a higher percentage of patients in the insufficient range for vitamin D (37.5%).

When we carried out the comparison of the values of 25HCC as a function of the level of spinal injury, we observed that the paraplegic patients had values of vitamin D indicative of vita-

min D deficit higher than the tetraplegics (69% vs 61.2% respectively).

In terms of the other parameter studied, the PTH, a prevalence of secondary hyperparathyroidism of 5.8% was found. We obtained a statistically significant inverse correlation between levels of PTH and those of 25HCC (r= -0.262; p= 0.007) (Figure 1).

Discussion

Patients with chronic spinal injury had a higher prevalence of fragility fractures, above all in the long bones, due essentially to the reduction in mobility, although there may be other mechanisms which contribute, such as hypovitaminosis D.

In fact, some studies carried out earlier describe the existence of hypovitaminosis D in patients affected by spinal injury. Thus, in a group of 100 military personnel with this pathology studied in a the Veterans Hospital in New York, Baumann et al. found a deficiency in vitamin D in 32% of patients affected both by paraplegia and tetraplegia. These were patients of both sexes, with an average age of 51 years and an average of 20 years since the acute spinal injury. The threshold chosen by the authors for the establishment of deficiency was 16 ng/ml, a value much lower than the currently recommended value of 30 ng/ml, which means that in applying the same cut off point, the prevalence of vitamin D insufficiency would be even higher. Similarly, Hummel et al., in 62 patients of both sexes with spinal injury, found hypovitaminosis D in 39% of cases[8], establishing the deficiency threshold at 75 nmol/L of 25HCC (equivalent to 30 ng/ml) which is precisely the figure currently accepted for the establishment of vitamin D insufficiently[13].

In our study we have applied this cut off point, 30 ng/ml of 25-hydroxyvitamin D, which establishes vitamin D insufficiency as those values of 25-hydroxyvitamin D lower than 30 ng/ml, and deficiency to those lower than 20 ng/ml. These cut off points have been suggested by various authors[14-17] and by the IOF in their position document[12]. On the basis of this, in Spain a high prevalence of hypovitaminosis D has been reported both in the general population and in older people[18,19], and more specifically, in various pathologies[20-22].

It is known that low levels of vitamin D favour the development of secondary hyperparathyroidism[13,18]. In our patients we found a negative or inverse correlation between levels of PTH and those of 25-hydroxyvitamin D, which although statistically significant, we consider to be weak (r=0.262). Not all patients with low levels of vitamin D had secondary hyperparathyroidism, which suggests that there are other factors which may affect its presentation.

The patients had normal renal function, estimated by the determination of blood creatinine and urea, and blood levels of TSH were also within normal levels.

Table 1. Baseline characteristics of the population studied

Variable	Value	Percentage (%)
Number	104	100
Men	77	74
Women	27	26
Cause of spinal injury		
Traumatic	90	86.5
No traumatic	14	13.5
Level of spinal injury		
Quadriplegia	41	39.4
Paraplegia	63	60.6
Functional status (Scale Asia)		
1 (Complete injury)	56	53.8
2 (Incomplete injury)	48	46.2

Table 2 Biochemical data for renal and thyroid functions and lipids (Mean ± typical deviation)

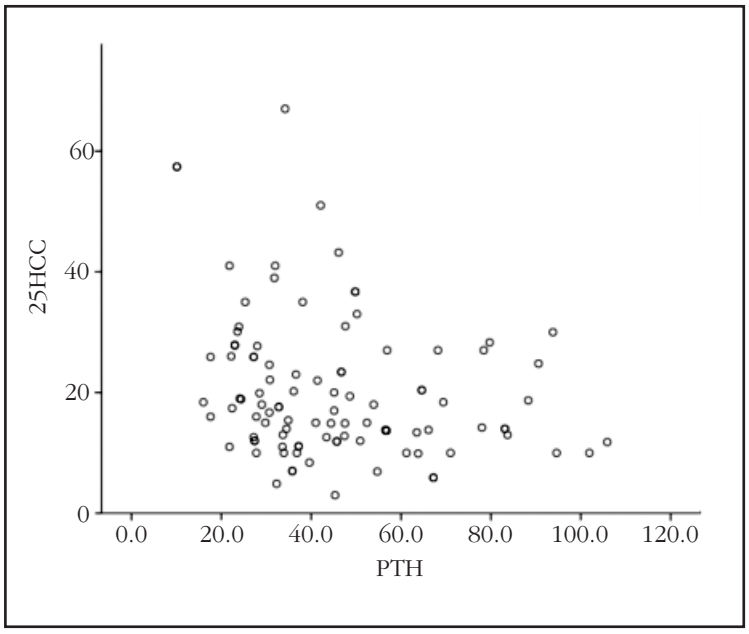
Variable (Units)	Value obtained	Values reference
TSH (UI/l)	1.8 ± 1.1	0.5 - 5
Urea (mg/dl)	29.2 ± 8.8	15 - 45
Creatinine	0.8 ± 0.2	0.8 - 1.4
Cholesterol (mg/dl)	191.1 ± 44.1	120 - 220
Triglycerides (mg/dl)	178.8 ± 9.5	30 - 200
HDL-Cholesterol (mg/dl)	47.1 ± 21.5	35 - 65

Table 3. Biochemical data related to bone mineral metabolism (Mean ± typical deviation)

Variable (Units)	Value obtained	Values reference
Calcium (mg/dl)	9.6 ± 0.4	8.8 - 10.4
Phosphorus (mg/dl)	3.3 ± 0.5	3.3 - 5
Total protein (g/l)	7.1 ± 0.4	6.2 - 8
Corrected calcium (mg/dl)	9.7 ± 0.4	8.8 - 10.4
Osteocalcin (ng/ml)	20.2 ± 9	14 - 46
TRAP (UI/l)	2.3 ± 0.6	0.1 - 3.9
Beta-crosslaps (ng/ml)	0.3 ± 0.2	0 - 0.5
P1NP (ng/ml)	43.3 ± 23.7	<36.4
PTH (pg/ml)	45.2 ± 21.1	15 - 88
25-HCC (ng/ml)	20.1 ± 11.6	>30

TRAP: tartrate-resistant acid phosphatase; P1PT: amino-terminal peptide of collagen type 1; PTH: parathormone; 25HCC: 25-hydroxyvitamin D.

Figure 1. Correlation between blood levels of 25-hyrdroxyvitamin D and PTH in patients with spinal injury.
(R= - 0,262; p=0,007)



The markers for remodelling were within the limits established as normal by our laboratory and which are shown in table 3. An increase in markers for resorption might have been expected, given that an increase in the destruction of bone in a state of immobility has been reported in both humans and animals[23-25], but we did not find this. It is possible that this was due to the long period of time since the spinal injury had occurred, since the increase in resorption and the loss of bone mass occurs in the first few weeks after the injury[26].

One of the limitations of our study is our having determined the 25HCC using IQL. It is well known that the standard reference model for the measurement of vitamin D is high pressure liquid chromatography (HPLC)[27], to which we did not have access in our Unit. Another limitation is that of having carried out only a descriptive study without using a control group with which to compare the results, but it should be taken into account that the levels set for the establishment of insufficiency and deficiency are already, practically speaking, a matter of consensus (30 ng/ml and 20 ng/ml of 25HCC, respectively)[11-15,17,20] as well as the upper limit of PTH, which has been established as normal at 88 ng/ml in our laboratory through other studies[28-31]. On the other hand, one of the strengths of the study is its sample size, 104 patients with spinal injury, the highest number found in the literature which we have been able to consult on this matter.

In conclusion, a high proportion of patients with chronic spinal injury had blood levels of 25-hydroxyvitamin D which may be considered as "insufficient", which is why we believe that it is necessary to generalise the study of vitamin D levels in these patients in order to detect and correct deficiencies when they are seen. We should also pose the question as to whether vitamin D supplements really reduce the risk of osteoporosis and fracture, which may be the objective of other studies.

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Table 4. Prevalence of normality, insufficiency and deficiency of vitamin D in patients with spinal injury as a function of sex

		Normal (>30 ng/ml)	Insufficiency (20-30 ng/ml)	Deficiency (<20 ng/ml)	Total
Men	% of the total	4	6	17	27
	number	3.8	26.1	26.2	26
Women	% of the total	12	17	48	77
	number	11.5	16.3	46.2	74
Total	% of the total	16	23	65	104
	number	15.4	22.1	62.5	100

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Risk of fracture associated with states prior to the diagnosis of diabetes mellitus type 2: Nested case-controlled study (DIAFOS cohort)

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Summary

Backgound: In phases prior to the diagnosis of diabetes mellitus type 2 there is an increased risk of cardiovascular disease, but it is not known if this is the case in relation to the risk of fractures.
Objective: To compare the prevalence of fracture in cases of diabetes mellitus and in matched controls.
Material and method: Nested case-control study in a population-based cohort. All patients diagnosed with type 2 diabetes in the period 2006-2011 were included, as were, for each of these patients, two control subjects of the same age, gender, and from the same medical centre, without diabetes. Any fractures, cerebro-vascular accidents and ischemic cardiopathy prevalent in these patients were identified using ICD codes 10. The prevalence of osteoporotic, major and hip fractures, and of cardiovascular disease at the time of diagnosis for the diabetic subjects, and on the same date for the matched controls, were calculated. Using conditional logistical regression the odds ratios (OR) were calculated, adjusting for body mass index, smoking, alcoholism, use of statins, cardiovascular disease and diabetic complications.
Results: 58,931 diabetic patients and 117,862 controls were identified. At the date of diagnosis the diabetic patients had a higher prevalence of cerebro-vascular accident (4.9% vs 3.5%; p< 0.001) and ischemic cardiopathy (8.1% vs 4.7%; p< 0.001). On the other hand, the prevalence of osteoporotic fracture (2.8% vs 2.7%; p= 0.22), hip fracture (0.4% vs 0.4%; p=0.63) and major fracture (1.5% vs 1.5%; p=0.97) was similar in both groups. The adjusted ORs were: 1.2 (CI 95%: 0.96-1.09), 1.08 (CI 95%: 0.90-1.28), and 0.99 (CI 95%: 0.91-1.09), respectively.
Conclusions: The type 2 diabetic patients had a higher prevalence of cardiovascular disease at the time of diagnosis. However, their risk of fracture was similar to the non-diabetic control subjects.

Key words: *diabetes mellitus type 2, osteoporotic fractures, cardiovascular disease, prevalence.*

Introduction

Diabetes mellitus and osteoporosis are two diseases which are highly prevalent in our environment. According to the estimates of the International Diabetes Federation, nearly 245 million people suffered from diabetes mellitus type (DM2) in 2006[1]. The prevalence of DM2 in adults in Spain varies between 12 and 15%[2]. Similarly, the overall prevalence of osteoporosis in our environment is 12.73% in women and 4.15% in men[3].

Although the relationship between diabetes mellitus and cardiovascular disease is well known, less data is available regarding the possible relationship between DM2 and osteoporosis. Different epidemiological studies indicate an increase in bone mass in type 2 diabetic patients while contrary to what would be expected, there is an increased risk of fracture, both in the femur and the vertebrae and other locations[4-6].

According to the natural history of DM2[7], patients may pass through an average of 5-10 years in a number of prior states characterised by alterations in carbohydrate metabolism: pre-diabetes. What is meant by pre-diabetes is those intermediate situations between normality and diabetes. Two states may be distinguished: impaired glucose tolerance (IGT), defined by the American Diabetes Association (ADA) as a value of glycemia of between 140 and 199 mg/dl 2 hours after a tolerance test with 75 g of glucose taken orally; and impaired fasting glucose (IFG) defined as a baseline glycemia of between 100 and 125 mg/dl. Although they should not be considered to be diseases, these pre-diabetic states are associated with an increased cardiovascular morbimortality[8], which is greater in patients with IGT than in those with IFG[9]. There is little data available on the risk of fracture in these states; the Rotterdam study[10] showed that those patients with IGT had a significant reduction in the risk of fracture.

As a consequence, this study was designed with the objective of analysing the prevalence of fractures at the time of diagnosis of DM2 in a population-based cohort.

Material and method

This is an nested case-controlled study in a population-based cohort (DIAFOS cohort). The data were obtained from the SIDIAP database, which contains the clinical information recorded by primary care doctors working in the Catalan Institute of Health (Instituto Catalán de la Salud (ICS)), the main provider of health services in Catalonia, as well as pharmacy invoice data, analysis data from reference laboratories and reports from hospitals in the public health system. This database includes information from approximately 5.8 million patients (approximately 80% of the Catalan population). After a quality control, information from almost 5 million people was available, demographically representative of this population. The quality of the information which SIDIAP contains has been validated[11]; earlier studies validated the records of incidents of fracture in comparison with classical cohorts[12] and the data related to the records of DM2[13].

From all the patients included in SIDIAP, those who had a diagnosis of DM2 in a period between 01/01/2006 and 31/12/2011 were identified, using the CIE codes 10. From all those diabetes-free (types 1 and 2) SIDIAP participants two controls per case were randomly selected, matched by year of birth, sex and health centre.

Data was collected regarding descriptive variables: age, sex, body mass index, presence of complications associated with diabetes mellitus (cataracts, nephropathy and diabetic neuropathy), smoking (smoker, non-smoker, ex-smoker), alcohol consumption (in average units of weekly consumption, classified as: low risk consumption, when in men it was between 17 and 28, or in women, between 11 and 17 units; risky consumption, when in men it was higher than 28 and in women higher than 17 units) and use of statins (ATC codes C10AA01 to C10AA08). The presence of ischemic cardiopathy (stable angina, unstable angina or myocardial infarction) and cerebrovascular disease (cerebral infarction or transitory ischemic accident) at the time of diagnosis with DM2 using CIE codes 10. In addition, all the clinical fractures recorded in the computerised clinical history prior to the diabetes mellitus diagnosis were collected by means of a review of CIE codes 10; The SIDIAP database does not contain imaging test information, for which reason it was not possible to radiologically confirm the fractures. Three different types of fracture were distinguished: osteoporotic fractures (in any location except fingers or toes, face or cranium); major fractures according to FRAX©[14] (hip, wrist, forearm, humerus and vertebrae) and hip fracture.

Statistical analysis

The characteristics of the population studied were described by means of a descriptive univariate analysis, calculating the mean and standard deviation for continuous variables and absolute frequency and percentage for categorical variables. To compare the prevalence of cardiovascular disease and of fracture in both groups the chi squared test was used. Using conditional logistic regression the unadjusted odds ratios were calculated for cardiovascular disease and fractures, and adjusted for the following confusion factors defined *a priori* according to the available literature and biological plausibility: body mass index, smoking, alcoholism, use of statins, cardiovascular disease, cataracts, nephropathy and diabetic neuropathy. All the statistical tests were carried out with a confidence (CI) of 95% and assuming a bilateral contrast.

The SIDIAP database provided purely observational data for this study. The SIDIAP data are totally anonymous and identified by an internal code created at the time of incorporation of the data, a fact which makes impossible the identification of the subjects included in the study. The approval was obtained of the local ethics committee for clinical research (CEIC IDIAP Jordi Gol).

Table 1. Baseline characteristics of the patients with DM2 (cases) and of the patients without DM2 (controls)

	Cases DM2	Matched controls	p
Number of patients	58,931	117,862	
Age in years. average (SD)	62.79 (11.97)	62.80 (11.97)	0.896
Men. N (%)	33,362 (56.6%)	66,724 (56.6%)	1
Women. N (%)	25,569 (43.4%)	51,138 (43.4%)	1
Overweight patients. N (%)	19,169 (32.5%)	31,962 (27.1%)	<0.0001
Obese patients. N (%)	26,472 (44.9%)	23,673 (20.1%)	<0.0001
Patients with moderate alcohol consumption and risk. N (%)	19,651 (33.3%)	28,298 (24%)	<0.0001
Active smokers. N (%)	10,228 (17.3%)	17,342 (14.7%)	<0.0001
Previous cataract patients. N (%)	3,849 (6.5%)	7,333 (6.2%)	0.012
Patients with prior renal disease. N (%)	6,546 (11.1%)	9,469 (8.03%)	<0.0001
Patients with previous diabetic neuropathy. N (%)	295 (0.5%)	128 (0.1%)	<0.0001
Patients receiving statins. N (%)	26,071 (44.2%)	29,535 (25.1%)	<0.0001

Results

58,931 patients were diagnosed with DM2 between 01/01/2006 and 31/12/2011, and 117,862 matched controls were selected. In Table 1 the baseline characteristics of both cohorts are described, which were, as to be expected, same sex and similar age, but with a higher proportion of patients who were obese or overweight, smokers and receiving statins among the diabetics.

In relation to the presence of cardiovascular disease at the time of diagnosis, 4,799 (8.1%) cases and 5,535 (4.7%) controls had ischemic cardiopathy prior to the index date (p<0.0001); and 2,895 (4.9%) and 4,125 (3.5%), respectively, had cerebrovascular disease (p<0.0001). The corresponding ORs were 1.79 (95% CI: 1.73-1.87; p<0.0001) for ischemic cardiopathy and 1.42 (95% CI: 1.36-1.49; p<0.0001) for cerebrovascular disease).

The prevalence of osteoporotic fractures, major and hip, was similar and without statistically significant differences (Table 2). Those patients with DM2 had at the time of diagnosis a similar risk of fracture to the non-diabetic controls (Figure 1). The adjusted ORs were: for osteoporotic fractures, 1.02 (95% CI: 0.96-1.09; p=0.46); for major fractures, 0.99 (95% CI: 0.91-1.09; p=0.93); and for hip fracture, 1.08 (95% CI: 0.90-1.28; p=0.39).

In analysing patient sub-groups according to their exposure to tobacco, use of statins or degree of control of the DM2 starting (defined by HbA1c <7% or ≥7%), no statistically significant differences in the risk of osteoporotic fractures, major or hip, were observed (Table 3). Those patients with an initial HbA1c above 7% have a risk of fracture of the femur very close to being statistically significant (adjusted OR=1.30; 95% CI: 0.96-1.75; p=0.09).

Discussion

The patients with DM2 have an increased risk of around 70% and 40% of ischemic cardiopathy and cerebrovascular disease respectively, compared with the matched controls. A recent meta-analysis[15] of patients with pre-diabetes (including patients with IGT and IFG) concluded that these have an increase of 26% in cerebrovascular disease (RR=1.26; 95% CI: 1.10-1.41; p<0.001). A study of patients with IFG[16], according to the criteria of the ADA (American Diabetes Association), found and increase in the risk of ischemic cardiopathy in women (OR=1.70; 95% CI:1.0-3.0; p=0.049).

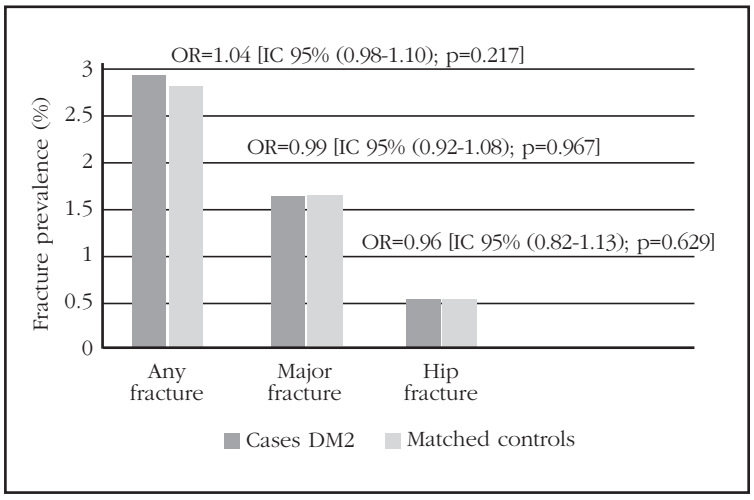
However, according to the results of our study, the patients with DM2 did not have a higher probability of suffering a fracture (major or hip osteoporotic fracture) than the rest of the population at the time of diagnosis with diabetes. This suggests that the increase in risk associated with DM2 does not appear in the initial phases of the disease but with the development of the disease itself.

The mechanism by which DM2 favours the appearance of fractures is not clearly defined, even though different substances have been described which have a crucial role in the pathogeny of this association[17-18]. In carrying out an analysis by sub-groups we have observed that those patients with an initial HbA1c above 7% have a risk of hip fracture at the limit of statistical significance. Previous studies[18] show that a degree of deficient metabolic control is associated with higher levels of sclerostin, provoking a risk of fractures. Furthermore, high levels of glucose leads to an accumulation of degradation products in the bone matrix, which results in bone which is biomechanically less strong[19]. Although our study did not have available data on these substances, it not

Table 2. Prevalence of earlier fractures groups in patients with DM2 and those without DM2

	Cases DM2	Matched controls	P
Osteoporotic fractures			
Number of patients	1,654	3,192	
Prevalence	2.8%	2.7%	0.224
Major fractures			
Number of patients	891	1,785	
Prevalence	1.5%	1.5%	0.967
Hip fractures			
Number of patients	232	482	
Prevalence	0.4%	0.4%	0.633

Figure 1. Risk of having a fracture at the time of diagnosis of DM2 (unadjusted OR) according to the location of the fracture



having found a higher incidence of fractures at the time of diagnosis of DM2 may support this hypothesis. The temporariness between the association of diabetes and fracture will be the object of a new prospective study in the follow up of the DIAFOS cohort.

As is to be expected, the patients with DM2 at the time of diagnosis have an increased risk of ischemic cardiopathy, as well as cerebrovascular disease, in comparison with the general population. Different epidemiological studies have shown that patients with glucose intolerance have a greater cardiovascular morbimortality[20]. Different mechanisms associated with hyperglycemia have been postulated as favouring arteriosclerosis, among which are endothelial dysfunction, oxidative stress and the formation of degradation products[21]. It is of interest to note that in the population of patients with DM2 in our study the proportion of patients who were overweight-obese and who were already receiving statins (indirect indicator of the presence of lipid metabolism disorder) was higher than in the controls. This may be explained by the fact that obesity and lipid metabolism disorders favour the appearance of carbohydrate metabolism disorder (impaired fasting glucose, glucose intolerance, diabetes or metabolic syndrome). In addition, the presence of a higher proportion of active smokers among those with diabetes type 2 may increase both the risk of suffering a cardiovascular event and fractures. The use of statins has been associated with a lower incidence of osteoporotic fractures[22], possibly due to the fact that it interferes in the same metabolic pathway, that of mevalonate, on which the bisphosphonates also act. A direct effect of statins has also been demonstrated *in vitro* in primary cultures of human osteoblasts[23]. However, its effects on bone are not very marked and do not, in our opinion, explain the lack of difference in the incidence of fractures. In any case, our analysis was adjusted for this possible confusion factor.

One of the limitations of our study is that the data comes from computerised clinical histories and, differently from the classical cohort study, may be underreported. This may result in bias in the random classification, which may lower the association between the predictor factors and the event of interest. In fact, the record of fractures in SIDIAP has been validated in comparison with the classical cohort studies and hospital discharge databases, and the data have a moderate sensitivity (nearly 70%) and a high specificity (>95%)[12]. On the other hand, this study has significant strengths, such as the high number of individuals, which allows the detection of statistically signifi-

Table 3. Analysis of risk of osteoporotic fracture, major and hip, by sub-group: exposure to tobacco, use of statins, and degree of initial metabolic control

	Adjusted OR [IC 95%]; p-value	
	HbA1c basal <7%	**HbA1c basal ≥7%**
Osteoporotic fracture	1.02 [0.94-1.10]; p=0.71	1.01 [0.90-1.14]; p=0.83
Major fracture	0.98 [0.94-1.10]; p=0.71	1.01 [0.86-1.18]; p=0.91
Hip fracture	0.97 [0.79-1.20]; p=0.77	1.30 [0.96-1.75]; p=0.09
	Never smoker	**Smoker/Ex-smoker**
Osteoporotic fracture	1.00 [0.93-1.09]; p=0.91	0.94 [0.76-1.17]; p=0.59
Major fracture	0.98 [0.89-1.09]; p=0.72	0.93 [0.67-1.28]; p=0.66
Hip fracture	1.06 [0.88-1.28]; p=0.53	0.81 [0.31-2.11]; p=0.67
	No prior therapy with statins	**Prior therapy with statins**
Osteoporotic fracture	1.08 [0.97-1.20]; p=0.16	0.98 [0.86-1.12]; p=0.81
Major fracture	1.05 [0.91-1.20]; p=0.54	0.99 [0.83-1.19]; p=0.93
Hip fracture	1.16 [0.88-1.52]; p=0.29	1.15 [0.77-1.71]; p=0.50

cant differences, even in cases where this is very limited. Therefore, we think that the lack of an increase in fractures at the time of diagnosis of DM2 is a consistent finding.

Conclusions

The DIAFOS cohort is made up of a population of patients newly diagnosed with diabetes mellitus type 2 in which the intention is to analyse the association between the diabetes and the presence of fractures. In this first analysis, using a nested case-controlled design within this cohort, we can conclude that at the time of diagnosis the diabetic patients did not have a higher risk of suffering a fracture than the general population, so that it appears to be the case that pre-diabetes does not cause an increased risk of suffering fractures, contrary to what happens with cardiovascular disease. So, these results do not support the need for specific evaluation of the risk of fractures in patients with a diagnosis of DM2 beyond what is normal in the general population.

Conflict of interest: The authors declare that they have no conflicts of interest.

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Divergent effects of TGF-β inhibition in bone metastases in breast and lung cancer

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Summary

Background: The objective of this study lies in the determination of the validity of transforming growth factor β (TGF-β) as a therapeutic target in models of metastasis deriving from different histological types of lung cancer.
Material and methods: 4-week-old immunodeficient mice inoculated with lung and breast cancer lines were treated with cytokine inhibitor peptide, control peptide or placebo. Weekly bioluminescence and microradiographic measurements were taken to determine the effects of the treatment on tumor burden and metastatic lesions in the long bones.
Results: Treatment with the specific peptide against TGF-β has a protector effect in the bone of animals inoculated with the breast cancer lines, unlike what happens in the control peptide and placebo groups. However, the anti-TGF-β treatment lacks the significant therapeutic effects on the bone metastases which develop in lung cancer bearing animals.
Conclusions: The role of TGF-β as a potential therapeutic target in bone metastasis is highly dependent on the histopathological type and subtype of tumor.

Key words: *TGF-β, bone, metastasis, animal models.*

Introduction

The skeleton is one of the preferred targets for tumour cells. Neoplasia of the breast, prostate, lung and myeloma very frequently generate bone metastases[1]. The prognosis for survival from a diagnosis of bone metastasis varies depending on the type of tumour. In patients with lung cancer with this type of metastasis, the average survival is measured frequently in months, being the lowest of all types of tumour with bone tropism[2]. Furthermore, on many occasions this phenomenon is detected at the time of diagnosis of the disease, a fact which contributes to this neoplasia being the primary cause of death from cancer[3].

The high frequency of bone metastasis may be explained by the endothelial fenestrations of the bone marrow, which could facilitate the establishment of the metastatic cells[4]. The bone is also a propitious environment for the development of these cells, since it is a medium rich in growth factors such as transforming growth factor β (TGF-β)[5]. The intracellular signalling route for this cytokine involves the phosphorylation of the Smad proteins, which ultimately allows the expression of the target genes in the nucleus. TGF-β has opposing functions in carcinogenesis. On the one hand, signalling through its receptor triggers an anti-proliferative response in conditions of oncogenic stress. This takes place through the induction of the expression of tumour suppressor genes such as the cyklin-dependent kynase inhibitors (CDKI) or the repression of oncogenes such as c-Myc and members of the ID family[6]. On the other, there are neoplasms which keep this pathway intact, evading this cytostatic response and simultaneously favouring tumour progression. Notable among other mechanisms is its contribution to the evasion of immunity mediated by the T CD8+lymphocytes[7] and angiogenesis by the induction of vascular endothelial growth factor (VEGF) and MMPs (matrix metaloproteases) in the tumour and the endothelium[8]. The tumour cell may also use TGF-β signalling for the progression of the metastasis in the bone microenvironment, given that this cytokine favours the expression of the protein related to the parathyroid hormone (PTHrP) or IL11[9,10]. These factors induce the expression of RANKL (ligand for receptor activator for nuclear factor κκB) in the membrane of the osteoblasts. The recognition of the ligand for the corresponding receptor in the mononuclear precursors entails their activation and the formation of the mature osteoclasts. The action of these cells promotes the release from the bone matrix of cytokines which prime the metastatic colonisation. Thus a positive feedback process or "vicious circle" is generated which magnifies the osteolytic effects.

The genetic or pharmacological inhibition of the signalling pathway of TGF-β has been shown to have therapeutic benefits in preclinical models. The expression of the dominant negative form of the TGFBR2 receptor in breast tumour cells or the use of the TGFRI SD-208 inhibitor in animals inoculated with melanoma cells with bone tropism has shown a reduction in metastatic lesions in the bone which these cell lines occasion[11,12]. Similarly, our group demonstrated this effect in a model of bone metastasis derived from a large cell pulmonary carcinoma line[13].

The main objective of this study is to assess the contribution of TGF-β to bone metastasis in models derived from other histopathological models of lung cancer such as adenocarcinoma or carcinoid. A model of bone metastasis derived from breast cancer was used as a control.

Material and methods

Cell culture

Tumour cell lines A549, H727 and MDA-MB-231 from lung adenocarcinoma, lung carcinoid and breast cancer, respectively, were used. The lines A549 and H727 were transfected with the retroviral vector SFG-NES-TGL (kindly donated by Dr Ponomarev), which contains the luciferase reporter gene. The cells were cultivated at 37°C and 5% CO2 in sterile conditions in RPMI (A549 and H727) or DMEM (MDA-MB-231) medium, supplemented with 10% FBS, 100 units/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen®).

Animals and intracardiac inoculation (I.C)

An injection of tumour cells was made into the left ventricle of 24 immunosuppressed mice, 4 weeks of age (Harlan Laboratories) in accordance with previous descriptions[14,15]. The cells were resuspended in PBS at a concentration of $2x10^6$ cells/ml. The animals were anaesthetised intraperitoneally prior to the inoculation with ketamine (65 mg/kg) and xylacine (2.5 mg/kg). $2x10^5$ cells (100 µl) were injected using a 29G calibrated needle.

All the protocols for working with laboratory animals were approved by the Ethics Committee for Animal Experimentation of the University of Navarra (CEEA).

Therapeutic regimen

Control peptides p41, or anti-TGF-β p17 or p144 (kindly donated by Digna Biotech as available) were used to assess this cytokine as a therapeutic target. Both had similar activity *in vivo*[13,16]. Five days after the inoculation of the tumour cell lines the animals were divided in 3 groups of 8 mice, each of which was to receive a daily dose, intraperitoneally, of 3.75 mg/kg of p144, control peptide (p41) or vehicle (physiological serum). The mice inoculated with the MDA-MB-231 line were divided equally, and from day 7 treated on alternate days with 2.5 mg/kg of p17 (similar activity to p144), control peptide p41 or vehicle.

The dose of p17 was chosen on the basis of its activity in previous studies[13,17], while that of p144 was higher to ensure that the results obtained *in vivo* with the lung cancer cell lines were not attributable to a low concentration of the peptide. The time period between the intracardiac inoculation and the start of the treatment had been established in earlier experiments, in which the time necessary for the cells to be detected in bone was determined, through bioluminescence or isolation of the metastatic cells.

The effect on the tumour was determined on a weekly basis through bioluminescence and/or osteolysis through X-ray analysis, looking for possible differences.

The duration of each experiment depended on the development of metastasis for each cell line.

Bioluminescence

D-luciferin (PROMEGA) was administered intraperitoneally at a concentration of 150 mg/kg to the previously-anaesthetised animals. After 5 minutes different images of bioluminescence were taken in a CCD chamber using the Living Image (IVIS® system, Xenogen) programme. The same programme was used to quantify the bioluminescent signal defining the lower extremities. From the data obtained was subtracted the luminometric value of a mouse not injected with cells (control). The values obtained were divided by the luminometric value of each extremity obtained before the start of treatment. The luminometric signal obtained appears superimposed on the rodent.

The cells transfected with SFG-NES-TGL, A549 y H727 vectors had a detectable signal. No tumour-related luminometric data from the animals inoculated with the MDA-MB-231 line was obtained, given that the vector with the luciferase gene with which it was transfected did not generate a signal, probably due to the methylation of the promoter[13].

Radiographic analysis

The X-rays were carried out under anaesthetic using a Faxitron® (MX-20) X-ray model. Film sensitive to this radiation (MIN-R, Kodak®) was used at 20kV for 20 seconds at 2x magnification. The radiographs were digitized at a resolution of 1200 ppi (Epson® Expression 1680 Pro). The area of osteolytic lesions was analysed with the computerised image analysis programme AnalySIS® (GmbH). The relative quantification of the metastatic areas was expressed as the percentage of the sum of the areas of lesion in the femur and tibia with respect to the total surface area of these long bones on the films.

Computerised microtomography (µCT)

Femoral and tibial joints representative of each experimental group were analysed in a microCT device (micro CAT II, Siemens® Preclinical Solutions) at 75.0 kVp and 250 uA. Each scan was carried out at a resolution of 10 µm. The two-dimensional images were reconstructed using a standard deconvolution procedure with a Shepp-Logan filter. For the reconstruction of the images the COBRA®_Exxim progamme was used. The images were stored in three-dimensional frames with a voxel size of 19*19*23 µm.

Statistical analysis

The SPSS 15.0 software programme was used to determine the statistical value of the results. A significance level of α=0.05 was used. Values of p lower than this limit were considered as significant (*). A single factor ANOVA analysis was carried out to study the metastatic area of the radiographs with multiple Tukey comparisons and the Kruskal-Wallace test followed by multiple comparisons with Mann-Whitney for bioluminescence. The p values obtained were adjusted with the Bonferroni method.

Results

The radiological analysis of the femurs and tibias of the animals inoculated with the MDA-MB-231 line showed a drastic reduction in the metastases of the animals treated with p17 in comparison with those treated with control peptide or placebo three weeks from the initiation of the experiment (Figure 1). In the animals inoculated with the H727 line, the bioluminescence images at day 22 showed a slight effect of p144 on the tumour load in the lower extremities of the animals treated with this peptide (*$p< 0.05$, Figure 2A). This effect lost its significance in the days following the experiment (Figure 2B). Similarly, the radiographical analysis of the long bones revealed a slight reduction in metastatic lesions in these mice which was not significant (Figures 3A and 3B). On the other hand, in the animals inoculated with the A549 line the p144 did not demonstrate any protective effect on the tumour load determined by bioluminescence (Figures 4A and 4B) or on the development of osteolytic lesions (Figures 5A and 5B). These results indicate that the prometastatic activity of TGF-β is highly dependent on each cell line.

Discussion

Metastasis involves the acquisition of new functions on the part of the cell in the primary tumour. These include motility and invasion, intravasation, survival in circulation, adhesion to the endothelium, extravasation and growth or colonisation of the target organs[18]. This requires a genetic and/or epigenetic programme –little understood at present– influenced by the selective pressure established in the tumour itself and its microenvironment. Therefore, the identification of the targets involved in metastasis is critical. Knowledge of the factors effecting this process could allow the development of new antimetastatic therapies which would have an impact on the quality of life of cancer patients. This fact justifies the development of models of metastasis which reproduce clinical reality. The model based on intracardiac inoculation recapitulates the final stages of metastasis: extravasation, homing, and colonisation of target organs. With this technique a high and rapid incidence of metastasis with reproducible results is achieved. This approximation has been used before with tumour lines of melanoma, prostate and breast[10,19]. The main limitation is the exclusion of the initial events of the metastatic cascade such as invasion, motility and intravasation towards the pulmonary parenchyma. The models which recapitulate these initial stages of metastasis such as orthopaedic injection have been successful for breast cancer. Here, an extirpation of the primary tumour after its

Figure 1. (Left) Radiographic analysis at day 21 of the metastatic area in the long bones of animals inoculated with breast cancer line MDA-MB-231. The osteolytic lesions (arrows) were evaluated after treatment with placebo (PBS), p17 (anti-TGF-β peptide) or p41 (control peptide). *p<0.05. (Right) representative X-rays of long bones of animals from each group

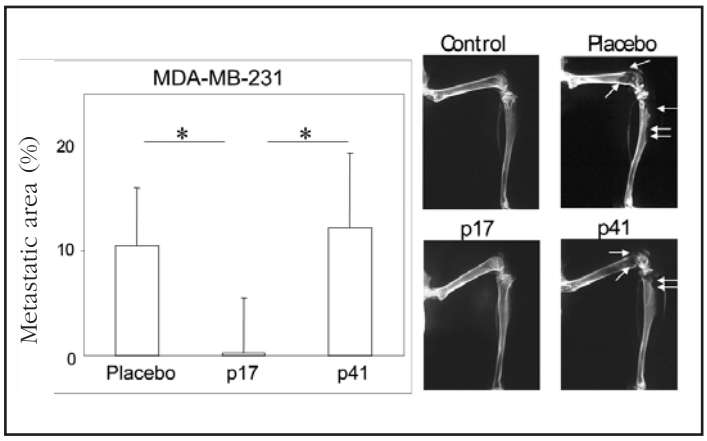


Figure 2 A. Analysis of bioluminescence in mice inoculated with the H727 line and treated with placebo, p144 (anti-TGF-β peptide) or p41. A moderate reduction in the tumour load 22 days after inoculation is observed in animals treated with p144 (*p<0.05; n.s., not significant in the later week). B. Images of bioluminescence representative of each group during the course of the experiment

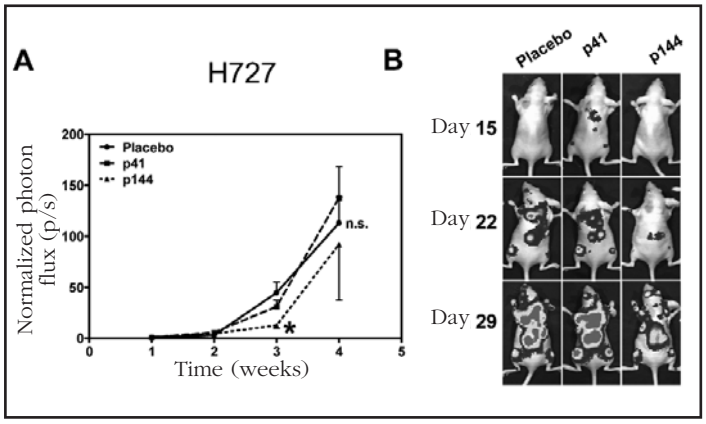
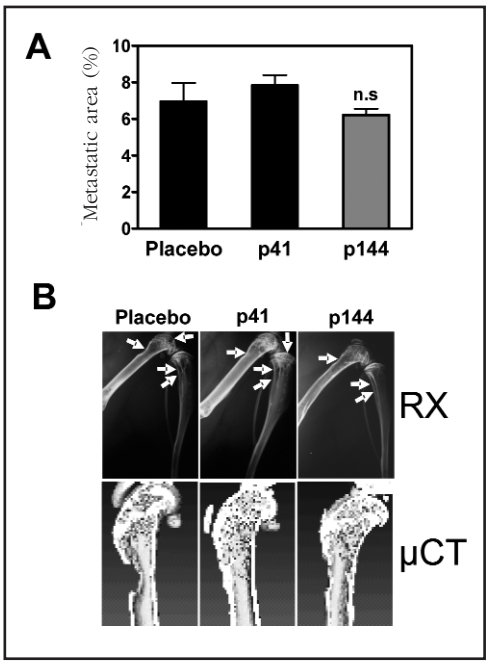


Figure 3. A. Radiographic analysis of the metastatic area in the long bones of animals inoculated with the pulmonary carcinoid line H727. B. Histomorphometric analysis: X rays (XR) and computerised microtomography (μCT)



growth is carried out to facilitate the later appearance of metastases at a distance[20]. However, this approximation is not viable in the case of lung cancer, since the death of the animals frequently ensues before the development of the metastasis, due to the rapid growth of the tumour. Furthermore, the frequency of bone metastasis in orthotopic models is low.

In spite of the importance of TGF-β in the bone microenvironment and in metastasis, its therapeutic potential should be treated with caution. Firstly, the systemic inhibition of this pathway may generate collateral affects, since signalling through this pathway is of vital importance in the homeostatic process of tissues. Secondly, the results obtained show that the effects of this pathway depend on the cellular context. Breast tumour cells evade TGF-β's inhibitory signals, keeping intact the route to the limbs which favours the prometastatic function of the cytokines. The great abundance of transcriptional inhibitors of the genes involved in the cytostatic response in these cells may account for this phenomenon[21]. This may explain the therapeutic improvement obtained in the model of bone metastasis of the MDA-MB-213 cell line. As occurs with breast cancer, pulmonary oncogenesis may involve the loss of the tumour-suppressant effects TGF-β[22]. Therefore, this cytokine may be of therapeutic interest for the treatment of bone metastasis of lung cancer. In agreement with these results, the use of a peptide inhibitor of TGF-β in a model of large cell carcinoma showed a reduction in bone metastasis[13]. However, the results shown in this work demonstrate that this effect on adenocarcinoma and carcinoid of the lung are of little or no significance. These experiments substantiate therefore the cell - dependent context of the TGF-β pathway. Furthermore, it is possible that other cytokines present in abundance in the bone, such as IGF-1[23], may constitute key elements for the progression of the vicious circle and consequent metastatic colonisation of bone. Future studies may determine other ideal targets for the development of antimetastatic therapies.

Figure 4. A. Analysis of bioluminescence in those mice inoculated with the A549 line treated with placebo, p144 or p41. (n.s., not significant). B. Images of bioluminescence representative of each group during the course of the experiment

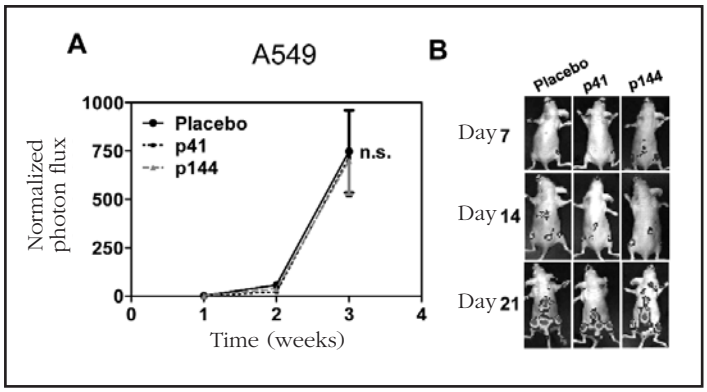
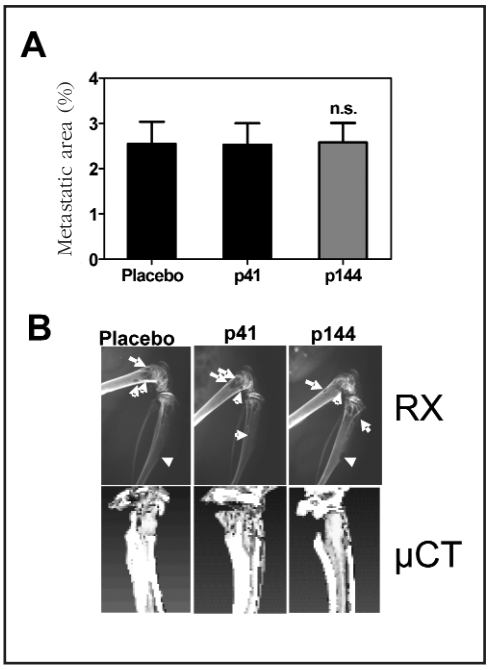


Figure 5. A. Radiographic analysis of the metastatic area in the long bones of animals inoculated with the pulmonary adenocarcinoma line A549. B. Histomorphometric analysis: XR and µCT



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SNPs in the 3'UTR of the RANK gene determine site-dependent osteoporotic fracture

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Summary

Objectives: The RANK/RANKL/OPG system is involved in the determination of bone mineral density (BMD) and bone microarchitecture. Our study seeks to evaluate if there are SNPs in the 3'UTR region of the RANK gene associated with osteoporotic phenotypes.

Material and methods: Seven genetic variants in 1,098 women from the BARCOS cohort were genotyped, and their association with BMD and osteoporotic fractures evaluated. An interaction with SNP rs9594738 in the RANKL gene which was previously associated with BMD was tested.

Results: None of the SNPs were associated significantly with BMD. SNP rs78326403 was associated with wrist/forearm fractures (Log-additive model odds ratio (OR)=3.12 [IC 95%: 1.69 ; 5.75]; $p=7.16x10^{-4}$), while SNP rs884205 was associated with fractures of the spinal column (OR=4.05 Recessive; [95% CI: 1.59 ; 10.35]; $p=8.24x10^{-3}$). Lastly, an interaction was detected between SNP rs9594738 from RANKL and rs78326403 from RANK on the presence of fracture (p=0.039). The analysis of the effects of combined genotypes rs9594738 and rs78326403 pointed to an increase in the prevalence of fractures in subjects with a greater number of unfavourable alleles, the ORs being 2.76 [95% CI: 1.30 ; 5.81]; p=0.007) and 5.14 [95% CI: 1.37 ; 15.67]; p=0.007) for 2 and ≥3 unfavourable alleles respectively, in comparison with none/1.

Conclusions: Two SNPs in 3'UTR from the RANK gene predispose to site-dependent osteoporotic fracture. An interaction with SNP rs9594738 from RANKL suggests an additive effect of BMD and bone strength.

Key words: *osteoporosis, fracture, SNPs, association studies.*

Introduction

Osteoporosis is one of the most common problems in postmenopausal women, with a significant economic impact on Western society[1,2]. According to the criteria of the World Health Organisation (WHO), osteoporosis is diagnosed non-invasively by measuring bone mineral density (BMD)[3]. Low impact traumas are the immediate consequence of osteoporosis and are a growing cause of hospitalisation, morbidity and mortality in old people[4]. However, the definition of these low trauma fractures as "osteoporotic fractures" may be misleading, since many of these patients have levels of BMD considered to be normal according to the WHO criteria[5,6]. To improve the identification of subjects at high risk of fracture, a series of studies[7-9] have proposed the clinical use of various predictors, including the WHO FRAX® algorithm[10-12], in place of only BMD. The identification of a number of predictors independent of BMD (such as family history of hip fracture) indicates that other factors, probably related to the microarchitecture or other elements of bone strength, play an important role in the definition of osteoporotic fractures.

The RANK/RANKL/OPG signalling system is fundamental to bone remodelling. The RANK ligand (RANKL) is a membrane protein of the preosteoblast cell or secreted by osteocytes which bond to the RANK receptor of the osteoclast precursor, thus promoting its differentiation and activation into a mature osteoclast. The osteoblast, in turn, also secretes the soluble protein OPG, which act as a decoy receptor and interacts with RANKL, impeding its union with RANK, thus inhibiting osteoclastogenesis. The equilibrium between OPG and RANKL and the union of the latter to its receptor RANK is key to determining the anabolic or catabolic state of bone. Thus the gene TNFRSF11A (locus 18q22.1), which codes for RANK, has a special significance in bone remodelling, since it determines the differentiation of the osteoclasts and their survival[13]. A change in this complex would provoke a deregulation of bone remodelling which may result in pathological states, such as osteoporosis.

Whole genome association studies[14-17] and studies analysing interaction of the SNPs of RANK/RANKL[18] provide evidence of the importance of the TNFRSF11A gene in the determination of BMD and the incidence of fractures. Changes in the 3'UTR region of a gene may affect its expression by modulating the bonding sites of microRNAs (miRNAs)[19]. Furthermore, it has been shown that miRNAs can specifically regulate osteogenesis[20]. Therefore, the hypothesis of our study was that genetic variants in the 3'UTR regions of genes important to bone metabolism could be associated with osteoporotic phenotypes.

The objective was to identify SNPs in the 3'UTR of the RANK gene as possible functional genetic variants which may affect the risk of fracture. On the other hand, a possible interaction between the SNPs associated with fractures within the RANK gene and the SNP rs9594738 of the RANKL gene (previously associated with BMD)[14] was also studied.

Materials and methods

Characteristics of the BARCOS cohort

All the patients of this cohort are postmenopausal women who made an initial visit to outpatients at the Bone Metabolism Unit of the Hospital del Mar-Parque de Salud Mar in Barcelona, Spain, due to the menopause[21,22]. The patients were registered consecutively, not selectively, and recruited prospectively, independently of their BMD values. Their age, weight, height, age at menarche, years since the menopause at the time of densitometry, months of breastfeeding and history of previous fractures (Table 1) were recorded. Women with metabolic or endocrine diseases, chronic renal insufficiency, chronic hepatic disease, cancer (except cancer of the surface of the skin), Paget's disease of bone, malabsorption syndrome or on treatment with hormone replacement, antiresorptive or anabolic agents, oral corticosteroids, antiepileptic drugs, lithium, heparin or warfarin, were excluded, as well as those who declined the invitation to participate and did not give their informed consent. Furthermore, those subjects with a history of early menopause (<40 years of age) were excluded from this analysis. The blood samples and written informed consent were obtained in accordance with the rules of the Hospital del Mar's Committee on Human Genetic Research.

Determination of BMD and fracture

Double energy X-ray densitometry, DXA (QDR 4500 SL, Hologic, Waltham, MA, EE.UU.), was used to measure BMD (g/cm^2) in the lumbar spine (LS) L2-L4 and in the femoral neck (FN). The technique had a coefficient of variation (CV) of 1.0% for the measurement of the LS and 1.65% for the FN. The vertebral and non-vertebral clinical fractures were recorded. The non-vertebral fractures were validated through medical records, and those of the spine through X-rays if there was a history of diagnosis of vertebral fracture, loss of height, or back pain. Vertebral fractures were defined as those which occur after the age of 45 years and due to a low impact trauma. Fractures of the face, fingers, toes or cranium were excluded. Vertebral fractures were defined in accordance with the semiquantitative criteria of Genant et al.[23].

Extraction of DNA

The buffy coats were obtained from 3 ml of blood collected in tubes of EDTA and stored at -20°C. The genomic DNA were obtained from the leucocytes through a process of salting out[24] or by using Autopure LS (Qiagen), a robotised workstation for the automated purification of genomic DNA, in the laboratories of the Biomedical Laboratory Support Services IMIM, Barcelona (Spain), and stored at -20°C.

Selection of the SNP and genotyping

The Ensembl (www.ensembl.org) and Entrez SNP (http:// www.ncbi.nlm.nih.gov/sites/entrez) databases were used to select the SNPs of 3'UTR of the RANK gene. Only those SNPs with a MAF >0.01 were included.

The genotyping for the polymorphisms were carried out at Kbioscience (Herts., England) using the Kaspar system v4.0 and the Kraken allele algorithm. For the quality control, 959 samples were genotyped for the SNP rs9594738 (around 11% of the total genotyping) using the SNPlex System (Applied Biosystem) of the CEGEN platform (Barcelona, Spain). There was 99.8% concordance between the results of the two techniques.

Statistical methods

The Hardy-Wienberg equilibrium (HWE) was calculated by means of the Chi-squared test and the p value calculated with the online calculator of Tufts University (Http://www. tufts.edu/ ~% mcourt01/ Documents/Court 20lab% 20 -% 20calculator.xls 20HW%).

To evaluate the association of the genotyped SNPs with BMD and fractures linear and logistic adjusted regression models, respectively, were used. The confusion factors considered for adjustment were the body mass index (BMI)[25] and the age for the association with fracture; and the age of menarche, years since the menopause at the time of densitometry months of breastfeeding and the BMI[25] for the BMD. With the hypothesis that the effect on the fracture phenotype of the different genetic variants of RANK may vary as a function of the bone architecture, the effect of the SNPs both in the predominantly trabecular sites of fracture (spine) and in sites with more cortical bone (wrist/forearm) were studied.

Separately, the interactions between rs9594738 of the RANKL gene and the SNPs associated with fracture of RANK on the introduction of multiplier terms in the regression equation, were tested.

All the analyses were of two tails and values of $p<0.05$ were considered to be significant. The statistical analyses were carried out using SPSS for Windows version 13.0 and version R 2.13.2 with haplo.stats, epicalc, SNPassoc, foreign, rms, and genetics packs.

Table 1. Baseline characteristics of the cohort BARCOS

Patient characteristics	Mean ± SD	n
Age of menopause (years)	48.46 ± 4.06	1096
BMI	26.16 ± 3.85	1088
Lactation (months)	7.73 ± 12.79	1091
LS densitometry age (years)	56.04 ± 8.49	1087
Years since menopause LS	7.59 ± 8.26	1091
BMD LS (g/cm²)	0.853 ± 0.15	1092
FN densitometry age (years)	57.89 ± 8.03	1003
Years since menopause FN	9.36 ± 7.91	1007
BMD FN (g/cm²)	0.683 ± 0.11	1009
Menarche, age (years)	12.89 ± 1.58	1081
Fractures:	152 (13.8%)	1098
Column		68 (44.7%)
Hip		8 (5.3%)
Wrist/Forearm		36 (23.7%)
Other		40 (26.3%)

Results

Seven genetic variants of 3'UTR of the RANK gene in the BARCOS cohort were genotyped (Table 2). All the SNPs, except rs72933640, were in HWE. However, the MAF of rs72933640 in BARCOS was similar to the MAF (0.108) published by the National Centre for Biotechnology Information (NCBI) for the EU population. The MAF for all the SNPs genotyped was > 0.01 in our population.

The SNPs rs78326403 and rs78459945 were in total linkage disequilibrium (D) (D'=0.999; R2=0.968). the latter, in having a lower genotyping efficiency, was eliminated from the statistical analysis.

None of the SNPs studied were found to be associated with BMD (data not shown). On the other hand, the SNPs rs78326403 and rs884205 were significantly associated with the prevalence for fracture in our cohort (Figure 1 and Table 2). For the SNP rs78326403, the log-additive model gave a value of p=0.053; OR=1.58 [95% CI: 1.00 ; 2.49]. For the SNP rs884205, the log-additive model gave a value of p=0.048 with OR=1.40 [95% CI: 1.01 ; 1.95], while the recessive model produced $p=4.9x10^{-3}$; OR=3.28 [95% CI: 1.51 ; 7.13]. Only SNP rs884205 exceeded the Bonferroni correction (p value for a significant association: $p=8.33x10^{-3}$) (Figure 1). No significant interaction was detected between them (p=0.87).

Then, both SNPs were analysed for their association with fractures of the spine or wrist/forearm separately(Table 3). SNP rs78326403 was associated with fractures of wrist/forearm (log-additive model, $p=7.16x10^{-4}$; OR=3.12 [95% CI: 1.69 ; 5.75]), but not with vertebral fractures (p=0.78, log-additive model). On the other hand the SNP rs884205

Table 2. List of the genotypes SNPs, MAF and p values for the association with fractures under a log-additive model (or other model if indicated)

SNP #	rs	Location[1]	Efficiency	MAF BARCOS	HWE	p	OR (IC 95%)
1	rs78622775	18:60052935	0.95	0.01[2]	0.73	(0.99)[c]	
2	rs12455323	18:60053891	0.94	0.32	0.90	0.82	
3	rs72933640	18:60054077	0.94	0.12	**0.005**	0.59	
4	rs78326403	18:60054440	0.95	0.08	0.27	0.053 **(0.022)[o]**	1.83 (1.11-3.02)
5	rs78459945	18:60054757	0.94	0.08	0.39	In DL with rs78326403	
6	rs72933641	18:60054804	0.95	0.13	0.07	0.57	
7	rs884205	18:60054857	0.94	0.19	0.32	**0.048 (0.0049)[r]**	3.28 (1.51-7.13)

[1]: ENSEMBL website, release 66, February 2012.
[2]: Due to low MAF for rs78622775, the only model possible is the codominant model. In the case in which a more significant p value is obtained in another model this is indicated between ( ). Bold type p<0.05; [c]: codominant; [o]: overdominant; [r]: recessive.

was associated with spine fractures (recessive model, p=8.24x10^{-3}; OR=4.05 [95% CI: 1.59 ; 10.35]), but not with fractures of wrist/forearm (p=0.66, log-additive model). In this case both SNPs exceeded the Bonferroni correction. After an additional adjustment for BMD, both associations continued to be significant (Table 3) although the nominal association between rs884205 and spinal fractures after adjustment for LS BMD did not exceed the Bonferroni correction (recessive model p=0.025).

The analysis of the interaction between the SNP of RANKL rs9594738, previously associated with BMD, and rs78326403, considering as a variable wrist/forearm fractures, produced a significant result (p=0.039). Taking into account fractures of the spine, there was no interaction between rs9594738 and rs884205 (p=0.39). Nor was there any significant interaction found when the variable studied was BMD. The analysis of the effect of the genotypes composed of the SNPs rs9594738 and rs78326403 pointed towards an increase in the prevalence of fractures in subjects with a high number of unfavourable alleles (Table 4). Due to minor or zero differences between carriers with no, or only one, unfavourable allele on the prevalence of fractures, both categories are combined. Similarly, due to the small number of patients with 4 unfavourable alleles (n=3), this category was combined with the group of 3 unfavourable alleles. In general, the comparison was carried out in the following way: 0/1 vs 2 and 0/1 vs 3 or more unfavourable alleles. The analysis of the effect of these compound genotypes on the prevalence of fracture suggest and additive effect with the corresponding adjusted ORs: 2.76 [95% CI: 1.30 ; 5.81], p=7.4x10-3; and 5.14 [95% CI: 1.37 ; 15.67]; p=7.5x10-3, for2 and ≥3 unfavourable alleles, respectively (Table 4).

Discussion

The equilibrium of bone remodelling is, in part, regulated by the RANK/RANKL/OPG system. It is for this reason that this system has been well studied in the field of osteoporosis. In the light of this growing interest in the miRNAs as an epigenetic regulatory element, this study was centred on SNPs, situated in the 3'UTR of the RANK gene, which may affect the bonding of miRNAs. 7 SNPs in this region have been genotyped, resulting in two of them having been associated with osteoporotic fracture. The databases available to date offer little information, most of them in silico and based on algorithms, in terms of the binding sites of miRNAs in RANK's 3'UTR region. These databases, furthermore, do not hold any information about the genomic sequences which the SNPs associated with fractures contain. Therefore we do not have data regarding miRNAs which can bind to the region which contains the significant SNPs, and therefore functional studies would be necessary to clarify this question.

In recent years there has been a deepening in the study of genomics and the events which occur from gene expression to the final protein, which is to say, all those steps and molecules involved from the point at which gene expression takes place in the primary miRNA up to the operative functional protein, passing through the whole process of regulation, both of transcription and of

translation. The microRNAs (miRNAs or miRs) form a part of this dimension in gene regulation. The miRNAs are short single-stranded RNAs, ssRNAs) of 19-25 nucleotides generated from endogenous transcriptions with a hairpin shape, and have been evolutionarily preserved. Their function is the post-transcriptional silencing of genes through pairing with target mRNA in the 3'UTR region, resulting in the removal of this mRNA and thus the repression of its translation. A particular miRNA may act on a number of genes, and various different miRNAs may act on the same gene. If the pairing between the miRNA and the mRNA is only partial, a translational repression and later degradation results. If the pairing between the miRNA and mRNA is perfect (or nearly so) removal and degradation occurs. The majority of the targets of the miRNAs identified are in the 3'UTR of the messenger RNAs, which include transcription factors, receptors and kinases. The miRNAs regulate the protein translation and the stability of the mRNA, and thereby can modify numerous pathways related to development and diseases. Different studies have shown that genetic variants in the sequences of the miRNAs, as well as in their target sequences in the 3'UTR of the genes, may alter the mechanism regulating gene expression, and as a consequence, lead to a range of pathologies[19]. Lei et al. identified 3 polymorphisms located in potential binding sites for 9 miRNAs in the 3'UTR of the FGF2 gene which were associated with BMD in the hip[26]. We have identified 2 SNPs in the 3'UTR region of the RANK gene, rs78326403 and rs884205, which are associated with osteoporotic fractures in the BARCOS cohort. In a later analysis, each SNP showed a stronger association as a function of the site of the fracture studied: rs78326403 was found to be associated only with fractures of the wrist/forearm, while rs884205 was only associated with fractures of the spinal column. Furthermore, adjusting the results for the corresponding BMD (FN-BMD for rs78326403 and LS-BMD for rs884205) did not change the association of rs78326403, while the association of rs884205 with fracture was attenuated. These findings suggest that different SNPs in the RANK gene, even situated in the same region, may have a differential influence in a particular fracture site (cortical vs, trabecular bone). These associations with different types of fracture suggest different mechanisms of regulation of bone metabolism in predominantly cortical bone and in bone which is predominantly trabecular. Other factors have also been shown to be capable of differentially regulating cortical and trabecular bone, such as the GH-IGF-1[27-30] axis or the gonadal hormones[31-34]. It should be mentioned that these hormones regulate bone remodelling by means of RANK/RANKL/OPG[35-39]. Therefore, it seems plausible that the different hormonal signals may effect their action through different elements of the RANK/RANKL system.

Figure 1. Results of the study of the association of the genotyped SNPs with the prevalence of osteoporotic fracture in the BARCOS cohort for all the statistical models, presented as $-\log_{10}$ (p value). In each of the graphics, the lowest threshold line represents p=0.05, and the highest line represents the p value necessary after the Bonferroni correction ($p=8.33x10^{-3}$)

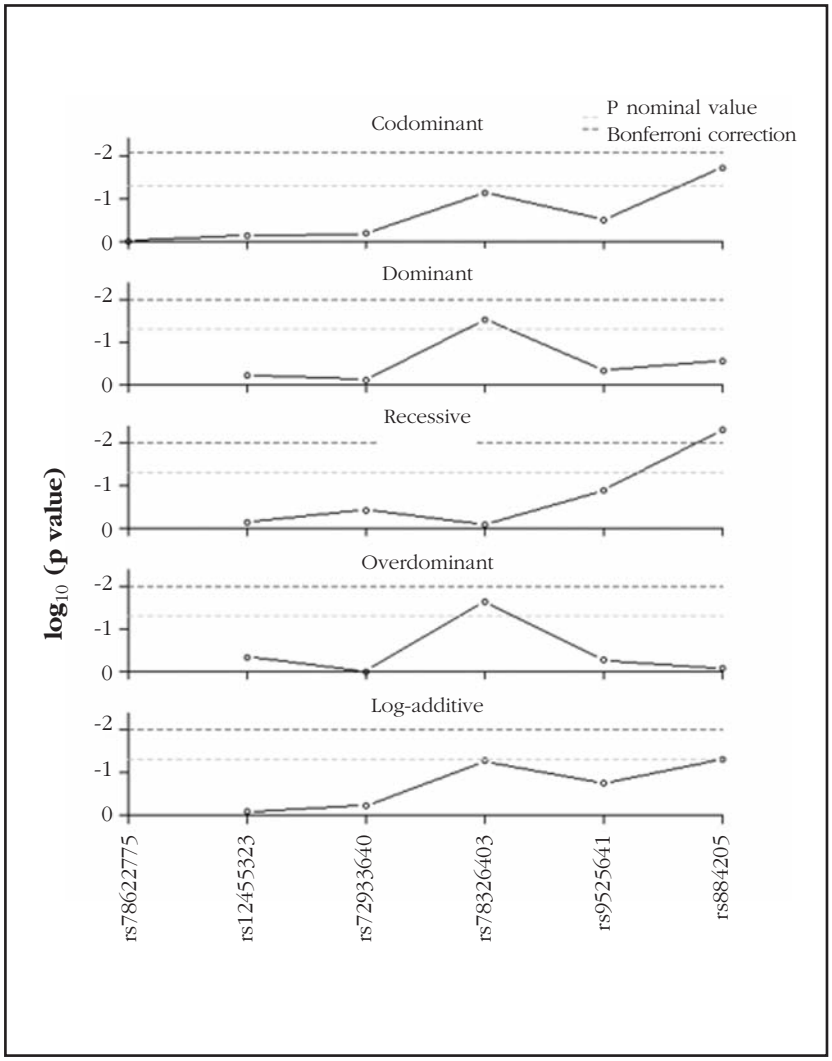

Table 3. Significant results regarding the association between the SNPs rs884205 and rs78326403 and the site of fracture

SNP	Site fracture	n	n fractures	p	OR	IC 95%	p[1]	OR[1]	95% CI[1]
rs78326403	Wrist/ forearm	1,033	34	$7.16x10^{-4}$ [a]	3.12	1.69-5.75	$5.8x10^{-4}$ [a]	3.21	1.74-5.94
rs884205	Column	1,029	62	$8.24x10^{-3}$ [r]	4.05	1.59-10.35	0.025 [r]	3.31	1.24-8.82

[1]: Result after correction for BMD: BMD in the femoral neck for rs78326403 and in the spinal column rs884205; [r]: recessive; [a]: log-additive.

Table 4. Analysis of the effect of the genotypes composed of the SNPs rs9594738 and rs78326403: 0/1 unfavourable alleles as a reference group vs 2 and ≥3 unfavourable alleles

Interaction	n unfavourable alleles	n	n individuals with fractures (%)	Grups	p	OR	IC 95%
	0	266	7 (2.6%)	Reference group			
rs9594738x	1	476	9 (1.9%)				
rs78326403	2	244	14 (5.7%)	0/1 vs 2	$7.4x10^{-3}$	2.76	1.30-5.81
	3	33	4 (12.1%)	0/1 vs 3/4	$7.5x10^{-3}$	5.14	1.37-15.67
	4	3	0 (0%)				

This is the first time that the SNP rs78326403 has been associated with fractures; SNP rs8844205 had previously been associated with osteoporotic phenotypes[16,40]. In a recent meta-analysis carried out by the GEFOS-GONOMOS consortium[41], which included the BARCOS cohort, rs884205 was associated with BMD, but not with fractures. This difference may be due to the heterogeneity between the different cohorts in the evaluation of the fracture on the part of the different groups of the consortium. A future replication should clarify the association of this SNP with the risk of fracture.

Finally, a significant interaction between the SNP rs9594738 of RANKL, which is associated with BMD, and rs78326403, suggests an epistatic effect between RANK and RANKL. In agreement with these results, in a study of the compound genotypes it was confirmed that there was an additive effect of the alleles of the two SNPs. So, the carriers of more unfavourable alleles have a higher risk (OR=5.14) of suffering fractures with respect to the carriers of the more favourable genotypes.

In agreement with our data, numerous genetic association studies have shown a different inheritability for BMD and fracture (or bone quality)[14-16]. However, it is widely known that these osteoporotic phenotypes are closely related, and our results reinforce this premise. Consequently, it is necessary to consider both low BMD and other additional measurements of bone quality or microarchitecture to evaluate with precision the risk of fracture in a clinical setting. These findings may be clinically relevant in the future for a more specific approach for different types of fracture, both to better understand their underlying mechanisms as well as to seek more specific therapeutic strategies. Our study has various limitations. The sample available is relatively small (1,098 women) having a limited number of fractures, and this reduces the statistical power of the study and, therefore, our ability to identify and analyse rare genotypes. Furthermore, our results could be specific to the population studied, which was limited to postmenopausal Caucasian Mediterranean women. Other studies in larger cohorts with different characteristics could determine if the associations which we have reported are replicable.

In conclusion, we have identified 2 SNPs in the 3'UTR of the RANK gene (rs78326403 and rs884205), which are associated with osteoporotic fractures. In our cohort, each of the SNPs is associated with a specific site of fracture. It was also found that there was a significant interaction between rs78326403 and an SNP (rs9594738) in the RANKL gene associated with BMD, highlighting the importance of BMD and microarchitecture as genetically determined predictors of the risk of fracture.

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

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Vertebral fractures as a debut to Cushing's syndrome diagnosed after a pregnancy

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Summary

The case is described of a 34 year old patient with Cushing's disease diagnosed as a result of having multiple pathological vertebral fractures after giving birth. The sudden appearance of acute fractures in five vertebral bodies, the phenotype characteristics of the patient and her medical history pointed to a diagnosis of Cushing's syndrome, a condition which rarely coincides with pregnancy. After a resection of the hypophysary adenoma and the start of treatment with teriparatide, the patient experienced notable clinical and densitometric improvement. This case demonstrates the importance of suspecting a bone metabolism disorder in the presence of pathological fractures in young patients, even more in certain states, such as pregnancy or lactation.

Key words: *Cushing's syndrome, osteoporosis, pregnancy, lactation.*

Introduction

Cushing's syndrome comprises symptoms and signs associated with prolonged exposure to inappropriately high levels of glucocorticoids. Excluding those cases due to chronic use of glucocorticoids, the great majority (60-70%) are due a hypophysary adenoma; this is what is known as Cushing's disease. Other causes are: tumours and anomalies in the suprarenal glands and ectopic secretion of ACTH.

Osteoporosis is very common in patients with Cushing's syndrome. The functional and structural deterioration of bone is a significant cause of morbidity and incapacity in these patients, who have a higher risk of, fundamentally vertebral, fractures.

On the other hand, pregnancy and lactation result in multiple changes in women, many of which affect the bone.

However, the combination of Cushing's syndrome and pregnancy is highly infrequent due to that fact that hypercortisolism usually coincides with amenorrhea and infertility.

Clinical case

The patient was a Spanish woman of 34 years of age, referred to the bone metabolism disease clinic due to having had several vertebral fractures after having given birth.

The patient had no family history of fractures. She confirmed having a sufficient daily intake of milk derivatives and low levels of regular physical activity. Of interest in her medical history were having had a transverse traumatic fracture of the patella three years earlier, with complications of pseudarthrosis. The patient had also been diagnosed with persistent polycystic ovarian syndrome due to amenorrhea 14 years previously, and had received continuous treatment with cyproterone acetate and ethenylestradiol, suspending treatment when trying for a pregnancy. After suspending the anovulatories the patient continued with her earlier amenorrhea without achieving gestation, which finally required techniques of assisted reproduction (*in vitro* fertilisation). The pregnancy was without complications (no gestational diabetes, and no hypertension or preeclampsia). Normal lactation was maintained for 5 months. The patient confirmed having gained weight in recent years, and significantly so after giving birth. A month after the birth persistent pain in the lumbar region started. After two months of lumbalgia resistant to treatment a magnetic resonance scan of the spine was performed, which showed evidence of fractures in various vertebral bodies, both dorsal and lumbar, with a reduction in height of the vertebral bodies being observed in T6, T8, T9, T11, T12, Li, L2, L3 and L5, with acute signs in T8, T9, T10, L1 and L3 (Figure 1). A physical examination showed facial rubicundity, accumulation of supraclavicular and retrocervical fat, with some cutaneous atrophy. The weight of the patient was 75.8 kg, height 1.61 metres, with a body mass index of 29.2 kg/m^2. Blood pressure was 150/95 mm Hg, confirmed on various occasions.

Notable from the general analysis were the following values: haemoglobin, 13.4 g/dl; sedimentation velocity in the first hour, 10 mm/h (normal, <25); 25-OH-vitamin D, 8.39 ng/ml (20-50); blood calcium 8.6 mg/dl; intact parathormone (PTH), 26 pg/ml (normal, 10-65); alkaline phosphatase 93 UI/l (normal, 38-126); carboxy-terminal telopeptide of collagen (CTX), 0.595 ng/ml (normal 0.064-0.548), amino-terminal propeptide of procollagen (P1NP), 38.7 ng/ml (normal, 10.4-62); urinary cortisol in 24 hours, 616.00 and 779.9 µg (normal, <200); creatinine in urine, 41.6 mg/dl; ACTH, 81.97 pg/ml (normal, 4.7-48.8); TSH, 0.45 µUI/ml (normal 0.465-468); and electrophoretic spectrum, unchanged.

The bone densitometry (double X-ray absorptiometry, DXA) was compatible with osteoporosis in the lumbar spine and osteopenia in the femoral neck: lumbar spine (L2-L4), 0,709 g/cm^2 (T-score = -3.26) and femoral neck, 0.683 g/cm^2 (T-score = -1.44).

On the evidence of ACTH-dependent hypercortisolism, and after a more than 50% decrease in blood cortisol after a strong dexamentasone suppression test, Cushing's disease was diagnosed. A cerebral magnetic resonance (MR) scan showed up a nodular image of4mm in the right adenophysis, on the superior and anterior margin (Figure 2). Treatment was initiated with fluconazol before surgery which improved pressure levels and provoked a small amount of the spontaneous menstrual bleeding. The patient underwent transsphenoidal surgery for resection of the microadenoma at 8 months postpartum.

Subsequent treatment with calcium and vitamin D supplements (1,500 mg a day of calcium carbonate and 400 mg a day of colecalciferol), daily subcutaneous teriparatide and hydroaltesone at subsitutive doses of 30 mg a day was recommended, as well as recommendations of a diet rich in dairy products and regular physical exercise. After surgery on the hypophysary adenoma the patient started to have spontaneous menstruations without treatment, with a normalisation of blood pressure, progressive disappearance of the Cushingoid phenotype which she had exhibited, and experienced a notable reduction in weight.

A year after the initiation of treatment with teriparatide she showed a notable improvement in DXA in both locations: lumbar spine (L2-L4), 0.837 g/cm^2 (T-Score = -2.03) and femoral neck 0.714 g/cm^2 (T-score = -1.14) as well as favourable changes in the analyses: alkaline phosphatase, 69 UI/l; 25-OH-vitamin D, 21.9 ng/ml; PTH, 31.4 pg/ml; CTX, 1.830 ng/ml; and P1NP, 286.9 ng/ml.

Discussion

Cushing's syndrome is defined as a combination of signs and symptoms derived from the prolonged exposure to inappropriately high levels of glucocorticoids.

Harvey Cushing was, in 1932, the first to postulate that the syndrome characterised by obesity, plethora, diabetes, hypertension, hirsutism, amenorrhea and osteoporosis, could be caused by hypophysary adenomas[1].

Cushing's syndrome is uncommon during pregnancy because the excess of corticoids and androgens suppress the secretion of gonadotropins, which brings with it ovarian and endometrial dysfunction. The most common cause of Cushing's syndrome during pregnancy is adrenal adenoma; however, there have been cases published in which the cause is hypophysary adenoma[2]. In addition, Cushing's syndrome during pregnancy carries a higher level of maternal morbidity, in up to 70% of cases[3], the most common complications being arterial hypertension and diabetes *mellitus*. Less frequent are osteoporosis, fractures, psychiatric disease and cardiac insufficiency. Therefore, this pathology is commonly underdiagnosed during gestation, it being confused with other conditions such as preeclampsia or gestational diabetes[4].

The physiopathology of steroid-induced osteoporosis (SIO) is complex, given that there are multiple factors involved, many of which have not been completely clarified[5]. There is a decrease in bone formation due to osteoblast and osteocyte apoptosis[6], as well as an increase in bone resorption due to the activation of the osteoclasts[7], which have a more prolonged useful life. The formation of new collagen is an inhibitor and the degradation of pre-existing collagen an accelerator[8]. The steroids also have effects on bone remodelling at the level of the basic remodelling unit, resulting in a reduction in average trabecular thickness and a lower degree of bone apposition. Furthermore, the steroids reduce the levels of insulin growth factor (IGF-1), growth hormone (GH) and sex hormones. With respect to calcium metabolism, they reduce intestinal absorption and increase the renal excretion of calcium[8].

Patients with Cushing's syndrome may have a decrease in levels of alkaline phosphatase and osteocalcin, which indicates the inhibitory effect of osteoblast function[9], as well as an increase in the parameters for bone resorption.

Bone fractures are present in 19-50% of patients with Cushing's syndrome, including Cushing's disease[10], and specifically, vertebral fractures in 16-20%[11]. In SIO the zones of greatest affectation are those with high trabecular bone content. This implies an increase in vertebral, rib and pelvic fractures.

SIO happens in two phases, an early rapid phase in which bone mineral density (BMD) is reduced by excessive bone resorption, and a slower progressive phase in which BMD decreases due to the damage to bone formation. However, SIO is reversible, the bone recuperation being gradual. It has been reported that the complete recuperation in BMD in patients cured of Cushing's disease may take more than 10 years[10]. Treatment with bisphosphonates and the active fragment of human parathyroid hormone (PTH 1-34) or teriparatide, may accelerate the recuperation of BMD in these patients. The drugs approved in Spain for the treatment of SIO are risedronate, zoledronate, and in cases where there is high risk of fracture, teriparatide. The bisphosphonates have an antire-

Figure 1. Magnetic resonance in the vertebral column

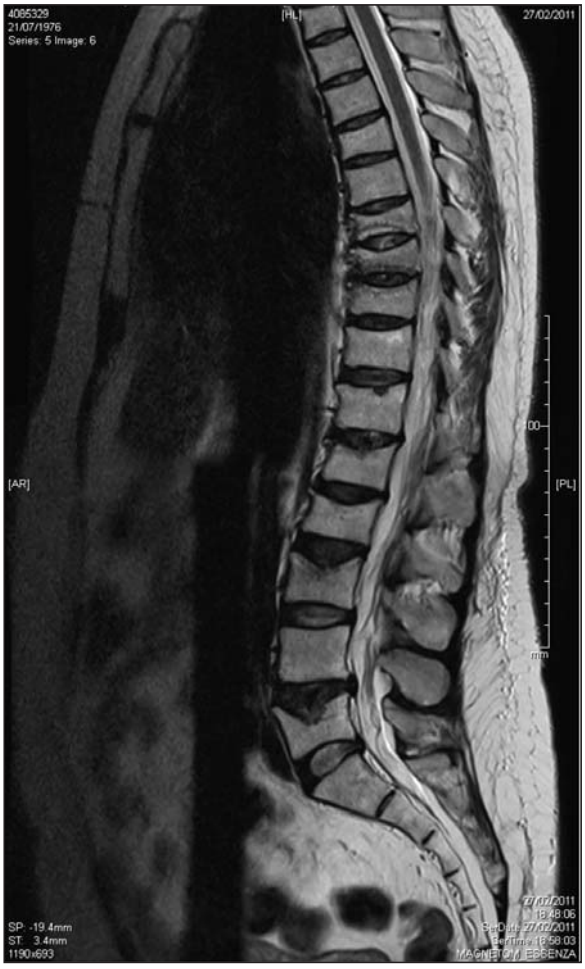
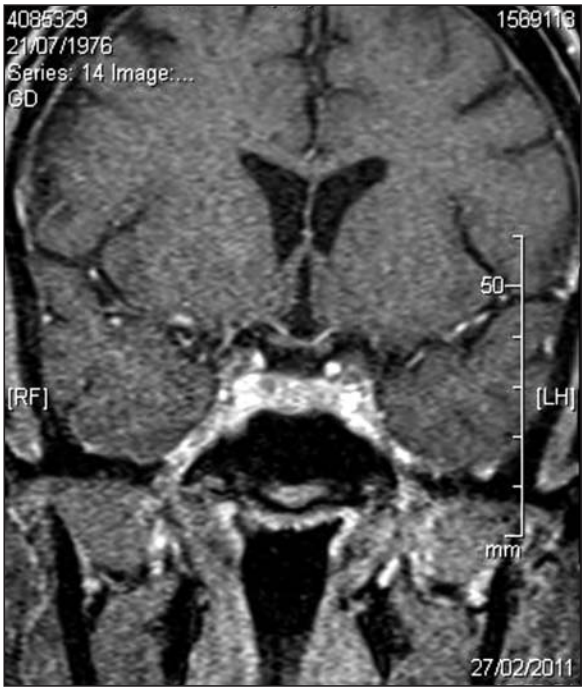


Figure 2. hypophysary magnetic resonance



sorptive effect by inhibiting the activation or recruitment of the osteoclasts. Risedronate, in being taken orally, is the drug of first choice in the treatment of this disease. Zoledronate is administered intravenously, once a year, which is more comfortable for patients and results in greater adherence to treatment. PTH 1-34 or teriparatide, obtained through recombinant DNA technology, has an anabolic action, given that it stimulates the formation of bone due to a direct effect on the osteoblasts, indirectly increasing intestinal absorption of calcium and increasing in the kidney the tubular resorption of calcium and the excretion of phosphate. It is also related with an improvement in bone mineral density and in bone quality[12]. It is approved for the treatment of established osteoporosis in postmenopausal women (capillary fragility fracture and BMD with a T-score value of less than -2.5) and in the treatment of steriodal osteoporosis, demonstrating an increase in BMD and a reduction in risk of fracture in these patients[13]. Its use is limited to a maximum of 18 month, and can then be replaced by an antiresorptive drug.

On the other hand, pregnancy is a state of hyperestrogenism which usually inhibits bone resorption[14]. However, on occasion, loss of bone mass and fractures have been reported. Osteoporotic fractures associated with pregnancy are characterised by the presence of pain in the lumbar region and the hip in the third quarter of gestation. Pubic and subcapital femoral fractures have been reported in pregnant women with osteoporosis[14]. However, rapid clinical and radiological resolution post partum is common[15]. In this case, the patient suffered multiple vertebral fractures several months after giving birth, which is not compatible with the transitory osteoporosis of pregnancy.

Maternal lactation may also affect a mother's bones. Some studies have shown that women may lose between 1 and 3% of their bone mass during lactation, due to the growing necessity of the newborn for calcium, the reduction in the production of estrogens and the increase in levels of proteins related with parathyroid hormones (PTHrP)[16]. However, the loss of bone mass which takes place during lactation is usually recovered in the first six months postpartum.

It is the combination of certain characteristics with makes this case interesting. Firstly, the appearance of a pregnancy without complications in a patient with Cushing's syndrome is unusual. Although this point may be arguable due to the absence of cortisol levels prior to the pregnancy, the appearance of prior weight-gain, defects in the consolidation of the facture of the patella and the recovery from sterility or amenorrhea after surgery, makes us think that Cushing's syndrome was present before the pregnancy. Secondly, the presence of a fulminant debut with multiple acute vertebral fractures is also uncommon in young people. Perhaps this point may be explained by the demineralising effect pregnancy and breastfeeding[16] has on bone previously affected by SIO. Thirdly, and lastly, what is interesting is the good response to teriparatide in a case of SIO.

There are multiple causes of secondary osteoporosis in young patients, many related to endocrinopathies. Therefore it is essential to suspect and include in the differential diagnosis Cushing's syndrome when faced with the presence of multiple pathological fractures, even in certain stages such as pregnancy and lactation.

The authors declare that they have no conflict of interest.

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Biomechanics and bone (& II): Trials in different hierarchical levels of bone and alternative tools for the determination of bone strength

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Summary

For a greater understanding of the mechanical properties of bone as a whole, it is first necessary to determine the behaviour of each of the components in an individual way at its corresponding structural level, as well as its overall involvement. This is the basis of the theory of hierarchical structure of bone, which involves its division into different structural levels. In this work we review, level by level, this hierarchical structure, reviewing the different mechanical trials which are applied to each of the structure. In addition, the methods for the determination of bone strength alternative to the classic mechanical trials are presented, which in recent years have been contributing significantly to the mechanical understanding of bone.

Key words: *biomechanics, bone tissue, bone strength.*

Introduction

Whatever the type of force to which bone is subjected *in vitro* the elastic modulus is always proportional to the bone mineral density (BMD), which means that the load necessary for the deformation of bone will be proportional to the degree of its mineralisation. However, bone with a very high BMD would imply a high degree of stiffness, which means it would be highly brittle. This shows that there are other factors in addition to its mass which influence the biomechanical efficacy of bone, such as the composition of bone tissue and its architectonic structure (macro- and microscopic), all these being grouped under the term bone quality. It has been estimated that the quantity of bone is responsible for 60 to 80% of its biomechanical strength, while the remaining 20-40% depends on bone quality, and it would therefore be a great mistake to underestimate its importance[1]. So it is vitally important to understand the contribution of each of the components of bone to its overall mechanical strength.

In the first section of this review[2] we provide an introduction to the field of biomechanics focussed on bone. We present the basic concepts of the issue and demonstrate the classic mechanical tests which have been used for some time in order to understand the mechanical properties of bone. However, in recent years advances in the field of biomechanics have gone much further, with mechanical strength of the different structural levels of bone being analysed separately, which is a great help in understanding the capacity of bone overall to support the loads to which it is subjected. In the second section we want to review the tests carried out at all structural levels. In addition, alternative techniques to the classical tests are presented which are increasingly used in the determination of bone strength.

The hierarchical structure of bone and its biomechanical properties

Bone is formed of an organic matrix composed mainly of type 1 collagen and a mineralised inorganic matrix (crystals of hydroxyapatite and calcium phosphatase). The collagen fibres which form bone are the result of the bonding by means of crossed links of a triple helix of chains of this material. This structure confers on bone its resistance to longitudinal traction and is largely responsible for its elasticity. The biomechanical properties which the collagen provides depend in turn on its utlrastructural characteristics, such as the quantity and orientation of its fibres or the stability of its links. In various pathological states these characteristics are seriously affected (mainly the stability of the links). On the other hand, the crystals are arranged in the spaces left in the organic matrix and are responsible for the stiffness of the bone and its resistance to compression, which means that these characteristics will be dependent on the quantity of the mineral, how densely it is packed and the arrangement of the crystals around the collagen fibres.

Due to its complex structure, in order to get to know and understand the biomechanical properties of bone its different structural levels need to be taken into account. Bone, in common with other biological materials, has what is known as a hierarchical structure composed of different levels as the scale varies. (Figure 1). These levels are defined in Table 1, according to the classifications established by different authors in recent years[3-6]. Each of these scales or hierarchical levels will have an influence on the biomechanical characteristics of bone.

Biomechanics of the whole bone

The mechanical behaviour of a material may be completely described by a group of material properties. However, the mechanical behaviour of a whole bone structure is much more complicated to predict, since it is the result of the material properties of each of its components and their geometric distribution in space.

Mechanical tests with whole bones or representative fractions of bone determine the properties of the bone as a whole, assuming that both the trabecular and cortical tissue can be modelled as a continuous structure, incorporating both its geometry and the properties of the materials of which it is composed. To be able to carry out this simplification, in doing so obviating the bone's anisotropy and heterogeneity, it is necessary that the test sample is significantly larger than the dimensions of its basic structural units. The biomechanical analysis of whole bone must always be accompanied by an analysis of its geometry. The mechanical behaviour of this type of sample is that which approximates most closely to the behaviour of bone *in vivo*; however, it is not appropriate to calculate material parameters at this level, since due to its complex geometry and the properties of the whole bone, it is not possible to identify changes in the microstructure or the extracellular matrix, which should be investigated at microscopic levels[5]. Nevertheless, tests of whole bone may be used to analyse the mechanical properties of the structural components, which is useful in the analysis of the effects which various factors, such as age, osteodegenerative diseases and their corresponding treatments, etc., provoke in the biomechanical properties of bone.

Much work has been carried out to understand the mechanical behaviour of whole bone, in which are used tests of compression and bending at three or four points and to a lesser extent, of torsion.

In the bending tests the measures consist of simple values of sagging and fracture loads, and stiffness (elastic zone incline). It is also possible to obtain a value for Young's modulus, but these calculations ignore the heterogeneity and the complex geometry of bone, since it is assumed that the bone is a perfect hollow tube, which means that the value obtained is simply approximated[7]. Nevertheless, it is the method most commonly used to estimate the mechanical properties of bone material in whole bone. Bone is more resis-

Table 1. Classification and definition of the hierarchical levels of bone proposed by different authors[3-6]

Hierarchical level	Principal components	Reference
Macrostructure	Cortical and trabecular bone	(Rho et al., 1998)[3]
Microstructure	Individual osteons and trabeculae	
Sub-microstructure	Layers	
Nanostructure	Fibrilar collagen and mineral components	
Sub-nanostructure	Molecular structure of the different elements	
Level 7	Whole bone	(Weiner and Wagner, 1998)[4]
Level 6	Cortical and trabecular bone	
Level 5	Osteons	
Level 4	Patterns of the fibres (mature bone vs interstitial bone)	
Level 3	Collagen fibres	
Level 2	Fibrils of collagen and minerals	
Level 1	Molecules	
Whole bone level	Whole bone or bone representative of both subtypes	(Hoffler et al., 2000)[5]
Architectural level	Cortical or trabecular bone	
Tissue level	Individual trabeculae and osteons	
Laminar level	Layers	
Ultrastructural level	Mineral and molecular components	
Macrostructure	Whole bone or bone representative of both subtypes	(An, 2000)[6]
Architecture	Blocks of cortical or trabecular bone	
Micrsostructure	Trabeculae and individual osteons	
Sub-microstructure	Layers, large collagen fibers	
Ultra- or nanostructure	Fibrils and molecules of collagen, mineral components	

tant to compression than to traction, and is even weaker in the face of shearing forces[8]. For example, when a long bone is loaded in a direction perpendicular to its longitudinal axis it suffers a bending load, since the impacted side is compression loaded, while the opposite side is traction loaded. As a result, the bone will begin to fail mechanically on the side opposite to the impact (the side subject to the traction), since this will reach its point of maximum resistance before the side subject to the compression.

Biomechanics of the tissue components

In terms of the structure of bone and its mechanical behaviour, we can see two subtypes of tissue: cortical bone and trabecular or spongy bone. The morphological differences between cortical and trabecular bone have significant biomechanical implications. The cortical bone has a higher elasticity modulus, which means that its stress-strain curve has a greater incline. This means that it is capable of supporting a greater degree of load per unit of surface area with a low strain index, which confers on it great stiffness. However, trabecular bone has a lower Young's modulus and biomechanically describes a flattened curve, which means that the supportable load per unit of surface area is lower, but with a higher strain index, which gives it greater flexibility (Figure 2).

Biomechanics of cortical or compact bone

The biomechanical analysis of cortical bone is carried out on cubes or cylinders which contain a sufficient number of Haversian systems and interstitial spaces to be considered representative. The upper limit for sample size will be determined by the anatomical region from which it is extracted[9]. The mechanical properties of cortical bone depend on the type of test to which it is subjected. In Table 2 are shown the values for strength and elastic modulus for human cortical bone[10-14]. The variations in the values are due principally to the anatomical region from which they come and the age of the sample.

Figure 1. Schematic representation of the different levels of hierarchical structure of bone

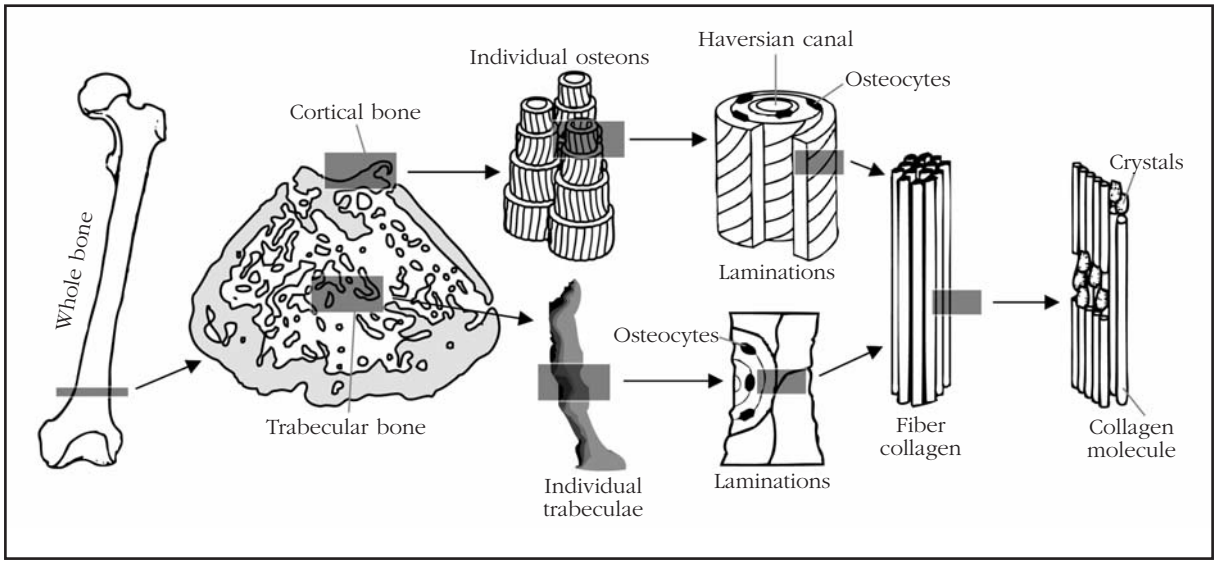
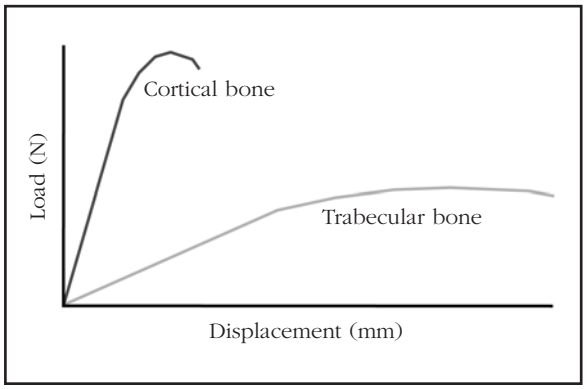


Figure 2. Load-displacement curve of biomechanical behaviour characteristic of the different types of tissue



Although the reference test for determining the biomechanical properties of cortical bone is the traction test, what is most frequently used is the bending test. The resistance to traction is less than the resistance to compression, and in the torsion test the value for the Young's modulus is much less than in the other two cases. Due to the longitudinal orientation of the collagen fibres and the osteons, cortical bone has a greater resistance to the application of longitudinal (0° inclination) than transverse (90° inclination) loads, and for intermediate inclination values, intermediate values of resistance will be obtained. In addition, its biomechanical strength longitudinally is also greater than that found with torsion loads. While the properties of a whole long bone are a function of its tubular form and its density, those of isolated cortical bone depend on its density and the orientation of the osteons. Due to this fact, the resistance values for cortical bone make up 60% of the strength of whole bone, which implies a greater mechanical strength for this tissue component[15].

The density of cortical bone depends on its porosity and the mineralisation of its material, and in human bone it has a value of approximately 1.9 g/cm^2, which is practically constant due to the fact that the cortical structure is quite compact[16]. It has been concluded that there is a positive correlation between cortical density and its biomechanical properties, such that if the former increases the latter improves. Porosity is defined as the relationship between bone volume and the total volume of the tissue, and is normally determined in a transverse section of cortical bone. Porosity and mineralisation explain 84% of the variation in stiffness in cortical bone[17], and experimental formulae have even been found which relate mineralisation with Young's modulus in such a way that an increase in mineralisation means a reduction in the elastic modulus[18].

The thickness and diameter of cortical bone are the main factors which affect its biomechanics. An increase in either of these characteristics results in an increase in bone strength. A long bone may be modelled as a cylindrical body, and, according to the basic laws of mechanics, resistance to the deformation of any cylindrical body subject to a force is directly proportional to its diameter. On the other hand the thickness of the cortical region and the quantity of bone mass are closely related, such that, with a constant bone mass, a variation in its distribution also modifies the bone's strength. The reduction in cortical thickness which happens with age, or in any osteodegenerative disease, has associated with it an increase in the risk of fracture.

Biomechanics of trabecular or spongy bone

In the case of trabecular bone the mechanical analysis is also performed using cubes or cylinders of this tissue subtype, of sufficient dimensions that the microstructural component does not influence the biomechanical properties. The structural properties of trabecular bone are usually determined using compression, traction or bending tests.

From the results obtained with these different tests it has been observed that trabecular bone, in the same way as with the cortical bone, has a greater resistance to compression than to any other type of load[19]. Its resistance in compression tests varies between 1.5 and 9.3 MPa, and the Young's modulus between 10 and 1,058 MPa, as a function of the region of the skeleton from which is comes. The density of human trabecular bone is approximately 0.43 g/cm^2. It has been concluded from experiments that both its strength and Young's modulus are a function of the square of its density, such that a small increase in density produces large increases in the two aforementioned parameters[20].

Table 2. Values of maximum resistance and elastic modulus of human cortical bone for the different types of mechanical tests[10-14]

Compression tests	Resistance	167 – 213 MPa
	Young's modulus	14.7 – 34.3 GPa
Tracción test	Resistance	107 – 170 MPa
	Young's modulus	11.4 – 29.2 GPa
Bending tests	Resistance	103 – 238 MPa
	Young's modulus	9.8 – 15.7 GPa
Torsion test	Resistance	65 – 71 MPa
	Young's modulus	3.1 – 3.7 GPa

The trabecular bone volumetric ratio (the quotient between the volume of trabecular bone and the total volume of the tissue, BV/TV) plays a very significant role in the mechanical stregth of bone. If the BV/TV reduces below 15% the structural integrity of the tissue is seriously endangered, having a much greater propensity to fracture. The number of trabeculae and their connectivity are also very significant in the biomechanical behaviour of spongy bone. The trabeculae are arranged vertically and horizontally, the latter arrangement being of vital importance to the bone's strength. It is possible to model spongy tissue as a combination of beams (horizontal trabeculae) and columns (vertical trabeculae), such that the former has the function of connection and securing the structure. A decrease in the number of trabeculae reduces strength, this reduction being more significant if it is the horizontal trabeculae which decrease. Reduced strength due to the narrowing of the trabeculae is reversible with the appropriate treatment. However, if the connectivity between the trabeculae disappears the loss of resistance becomes irreversible, since the original elasticity cannot be restored. Therefore, a structure with a greater number and thickness of, and connectivity between, trabeculae will be stronger than another with a lower number of trabeculae, less thick and with greater separation, even though both have the same bone mass.

The orientation of the trabeculae defines the degree of anisotropy. There is a correlation between the risk of fracture and the anisotropy of bone which is not dependent of the trabecular mass. The trabeculae are oriented such that they are stronger in the direction in which they normally support load, so resulting in heterogeneity or anisotropy in its structure. Therefore, if a region normally supports longitudinal loads (such as, for example, the femoral neck) its trabeculae are arranged geometrically so that it can better support these forces, and it is more resistant to loads in this direction (compression load), but there is a high risk of fracture with a load in another direction (for example, a transverse load due to a fall). Cortical bone also has an anisotropic behaviour due to the arrangements of the Haversian canals, but its mechanical impact is much less than is the case with spongy bone.

Biomechanics of osteons and individual trabeculae

The biomechanical analysis at this level describes the material properties of the tissue independently of it geometry, since it is carried out on samples small enough that the bone architecture does not have an influence on the result. In the case of cortical bone, the tests are carried out on a block of a few osteons and even only one, while for trabeculae bone a bundle of trabeculae would be used without its typical porous architecture, since in a sample of greater size the geometry would play a significant role in its biomechanical properties.

The use of nanoindentation tests for the analysis of the mechanical properties in very small samples has been developed in the last decade, allowing an in-depth analysis of structures such as trabeculae or individual osteons[21]. The technique of nanoindentation uses a rigid indentor with the aim of pressing on the surface of the material under test, thus provoking a local deformation of this surface. The force applied and the depth to which the indentor is applied is recorded both during the application of the force and once the sample is released, thus generating a load-displacement curve from which can be obtained the properties of the material (Figure 3).

The nanoindentation test equipment normally measures the force electromagnetically or electrostatically and the displacement by means of a capacitive sensor or a laser device. These methods allow the measurement of a force of between 1 and 500,000 μN, and a displacement of between 0.2 and 20,000 nm[22].

To perform an analysis of the osteons these first need to be isolated. Although it is possible to isolate a single individual osteon, their shape results in erroneous mechanical tests and the impossibility of comparing results. Therefore, the best option is to obtain, using a microtome which

continually refrigerates the bone, a sample in a defined shape: a cylinder which best represents the properties of the osteons. The isolation of the osteons is a complex process, in which the orientation of the laminations, their mineralisation, the distance between the vascular canal and the external surface, etc., are taken into account[17].

The osteons in human bone may be classified as a function of the orientation of the collagen fibres in the layers of which they are composed. When the collagen fibres of all the layers which form the osteon are longitudinally oriented one refers to these as longitudinal osteons. If the fibres of one layer are longitudinally oriented and those of the adjacent layer transversally, one refers to these as alternate osteons. Much less common is a third type of osteon in which the collagen fibres are oriented transversally, which are called transverse or circular osteons. Tests of compression, traction, bending and torsion are used to study the mechanical properties of the osteons, in addition to the so-called pin test, commonly used in tubular material mechanics. The longitudinal layers better resist traction and torsion, while transverse layers offer better resistance to compression, bending and shearing loads. In addition, it has been confirmed that the distribution of the layers in the osteons of the long bones is not random, but that there is a high incidence of longitudinal layers in the parts of the bone which support traction loads, and a high incidence of transversal layers in the sections which principally support compression loads[23-34]. No effects of age, gender or body mass index have been found on the elastic modulus or the toughness of the layers[21,31,32,35], from which it may be deduced therefore that the elastic modulus and the toughness of the bone matrix is independent of these variables, which means that the reductions in the mechanical integrity of the whole bone could be due to other factors, such as changes in the mass and organisation of the tissues[31]. Most of the studies which analyse the mechanics of trabecular bone use samples of sufficient size such that the biomechanical properties are influenced by the trabecular architecture as well as by the material properties of the bone. Traditionally, trabecular bone is considered to be like a more porous cortical bone, with the assumption that it would have the same elastic modulus, but in reality, in order to understand the mechanical impact of trabecular tissue itself it is necessary to carry out tests with individual trabeculae. As in the case of individual osteons, the mechanical analysis of the trabeculae is a complicated process which even requires the design of specific equipment. Three point bending tests[36,37] and traction tests[38,39] have been carried out. In the last few years advances in computerised microtomography have allowed models of individual trabeculae to be obtained, which are subsequently analysed by means of finite elements analysis[40]. The result show that the Young's modulus of trabecular bone taken independently is considerably lower than that of cortical bone, probably due to the lower degree of organisation which the former displays. Recently, two research groups have independently analysed, using computerised microtomography, samples of human trabecular bone taken from different parts of the anatomy, carrying out a complete decomposition of the samples on individual plates and tubes and calculating their contribution to the elastic modulus by means of finite element analysis. The results obtained show a predominance of longitudinal plates and transverse tubes in the three anatomical zones, and that the axial loads on the trabecular bone are largely sustained by the trabecular volume axially aligned. In addition, it is suggested that the trabeculae in the form of plates dominate the overall elastic characteristics of trabecular bone[41-46].

Biomechanics of the bone molecular components

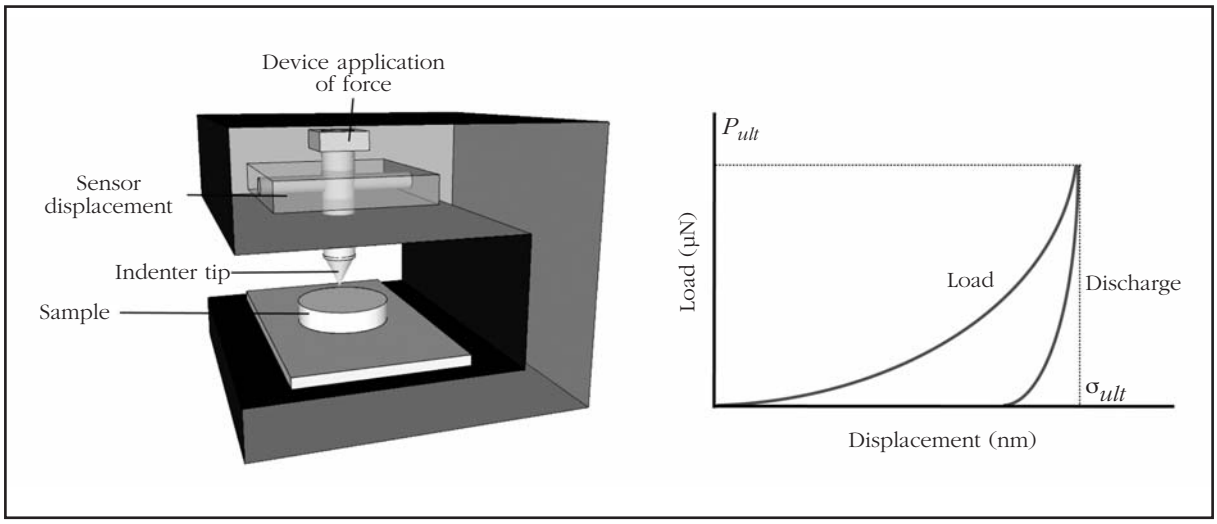
Bone at a molecular level is composed of proteins, glycoproteins and minerals, a composition which is known as an extracellular matrix. At this level it is interesting to study the mechanical properties of the collagen fibrils and the mineral components. The heterogeneity of the matrix makes the biomechanical analysis at this level yet more difficult, and the influence of the variations in the structure of the components is not known at present.

In 1997 Luo and collaborators presented a study in which the stiffness of the collagen molecules obtained from procollagen type 1 (which does not form intermolecular bonds) was measured using a system of optical tweezers and optical microscope[47]. Almost a decade later, using an electromechanical device, measures of resistance to traction, stiffness, and behaviour under fatigue of a collagen fibril were presented, while demonstrating for the first time its stress-strain curve[48]. A new experimental technique using atomic force microscopy and scanning electron microscopy have been used to manipulate and measure the mechanical properties of individual collagen fibrils in bone tissue. The stress-strain curve of the individual fibrils under traction stress shows an initial region of linear deformation for all the fibrils, followed by the non-homogeneous deformation above a critical deformation. This non-homogeneous deformation suggests possible changes in the mineral composition within each fibre[49].

The intrinsic mechanical properties of hydroxyapatite crystals have been determined by nanoindentation techniques. The basal faces of the crystals have a greater toughness and elasticity modulus than the lateral faces, but the latter are stronger. These results suggest that the crystals have a lower propensity to cracking and better resist microfractures on the lateral faces, which evidences the anisotropy of the hydroxyapatite crystals, which could have implications for the anisotropy observed on a larger scale[50].

The mechanics at a molecular level are influenced by all types of chemical interactions and unfortunately to date it has not been possible to carry out reliable and reproducible biomechanical

Figure 3. Schematic representation of the different components of a system of nanoindentation and of the typical load-displacement curve obtained in this type of test, in which are clearly differentiated the cycles of load and discharge. P_{ult} corresponds to the maximum load and σ_{ult} to the maximum displacement. The stiffness can be obtained from the tangent of the discharge curve



tests at this level. The methods of in situ analysis which combine high resolution tools for structural determination, such as X-ray diffraction, with micromechanical tests are starting to provide information regarding the real deformation which takes place at the molecular level and at the level of the mineralised and non-mineralised collagen fibrils[51,52].

Biomechanical techniques alternative to classical tests

Qualitative ultrasound analysis (QUS)

It has been a while since ultrasound techniques started being used for the evaluation of the mechanical properties of bone[53,54]. These techniques present various advantages over the classical mechanical tests in the determination of the elastic properties of bone, since samples which are very small and of different shapes can be used. Although qualitative ultrasound analysis does not produce an image of the structure of the bone there is real evidence that the QUS measurements may provide information related to the structural organisation and material characteristics of the tissue[55]. The advantages of QUS lie in the fact that no exposure to radiation is involved, as well as it being carried out using relatively cheap and portable systems. On the other hand, its principle inconvenience is its lack of sensitivity, which means that is it currently relegated to being used as an auxiliary tool in the diagnosis of osteoporosis, which is subsequently confirmed using bone densitometry (DXA). However, it is very useful in research work[56-59].

Finite element analysis (FEA)

Mechanical analysis using numerical simulation, and specifically the finite element method, has become a tool of great value when studying the biomechanical response of bone under various load conditions. The first step in carrying out a finite element analysis is the acquisition of images of the anatomical area or bone sample, normally using computerised microtomography (CT) or nuclear magnetic resonance (MRI). The sets of images obtained are processed by means of complex algorithms and sophisticated software tools with the aim of obtaining a mesh or model of the finite elements of the selected volume of interest. With these models it is possible to carry out both a morphological analysis of the structure and a simulated biomechanical analysis which will provide data on the strength and Young's modulus of the object analysed[60]. The most common FEA is the static linear analysis which calculates the mechanical resistance to static loads (which do not vary with time) and which assume that the material is isotropic and homogeneous. However, the development in recent years of technologies which allow the acquisition of high resolution images of the bone (micro-CT, HR-MRI, etc.), along with the use of new algorithms which represent the structure of bone with greater precision, has allowed the creation of models with which loads on the tissue and their anisotropic elastic properties[61] can be calculated. The FEA provides ever more precise data, becoming a powerful tool for the understanding of the biomechanical behaviour of bone, and one of the most used in the last few years[62-64].

In 1998 the first device to carry out mechanical tests for compression and traction from within computerised microtomography equipment appeared, so that the test could be followed step by step through high resolution images[65]. The authors called this technique Image-Guided Failure Analysis (IGFA). IGFA is very useful in the biomechanical analysis of samples of trabecular bone since it allows the observation of the progression of a fracture, monitoring its initiation and its

advance, while determining the influence of the microarchitecture of the sample, allowing knowledge of the microstructural properties local to the fractured areas as opposed to those areas remaining intact[66,67]. Recently a similar device has been developed to carry out torsion tests[68].

Nazarian and collaborators[69] concluded that 76% of the samples of trabecular bone from human lumbar vertebrae analysed with IGFA had minimum values of BV/TV, connective density and anisotropy in those regions in which mechanical failure had occurred with respect to the intact regions, with no significant differences being observed for other microstructural variables such as the number of trabeculae (Tb.N) thickness (Tb.Th) or trabecular separation (Tb.Sp), etc. On the other hand, our research group, in samples obtained from human osteoporotic femoral heads[70] found that the regions of fracture had worse values for all microstructural variables analysed, except for the degree of anisotropy. This reflects the fact that the region in which the failure occurs contains fewer trabeculae, of less thickness and which are less interconnected than the region which remains intact after the test, although in both the trabeculae are oriented in a similar way. In addition, in the region of the fracture tubular trabeculae are prevalent (theoretically less resistant to fracture) as opposed to trabeculae in plate form, which are more abundant in the intact region. The degree of correlation between σ_{ult} and a linear combination of microstructural variables (BV/TV, Tb.Th and trabecular pattern factor Tb.Pf) improves significantly when, instead of using the average values for the whole structure, the values for the region in which the fracture originated are used.

Thanks to this technology it has been possible to observe the different mechanisms of fracture. So, when a compression force is applied to trabecular bone, the structures in plate form fail preferentially on bending, starting in an area of the plate already perforated. In the case of structures in bar form, buckling is the predominant form of collapse (manifested by significant transverse displacement in the main direction of compression).

Conclusions

The complex mineralised organic matrix which constitutes bone tissue is hierarchical, in different structural levels which will define the mechanical properties of bone. Each of these hierarchical levels contribute in a different way, and to a different extent, to the overall mechanical behaviour of bone, and this needs to be taken into account when studying its biomechanical properties.

Many studies are being carried out nowadays on the different structural levels, and every day there are greater advances in the understanding of the behaviour of each of these structures, both individually and when combined in the tissue as a whole. Techniques alternative to the classical mechanical tests are helping extensively in the execution of this objective. Among these alternative methods there are non-destructive techniques such as FEA and QUS, which permit the repetition of the test as many times as may be necessary, and the changing of variables as and when required, which opens up great possibilities in the field of biomechanics.

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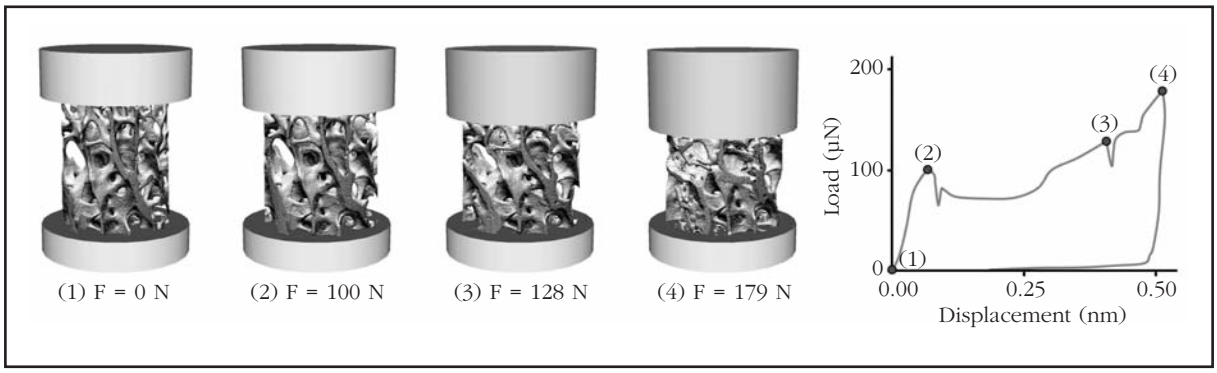
Figure 4. Three-dimensional representation of the initiation and progression of the mechanical failure due to compression in a cylinder of trabecular bone from a osteoporotic human femoral neck, obtained using the IGFA technique, which permits scans of the test sample with micro-CT equipment while the mechanical test is being carried out. The load-displacement curve shows the points at which each of the scans was carried out



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Osteoporosis and osteoarthritis: two mutually exclusive diseases or two related entities?

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Summary
The possible association between osteoporosis and osteoarthritis represents an ongoing matter of debate. It was considered, for decades, that both diseases were mutually exclusive due to the anthropometric characteristics and the difference in bone mass that patients with osteoporosis and osteoarthritis often present. However, in recent years, it was pointed out that both processes can coexist, and even that they may have a direct relationship. In this paper we review some aspects of the association between both diseases from a temporal perspective.

Key words: *musculoskeletal diseases, osteoarthritis, osteoporosis.*

Introduction

Osteoporosis and arthrosis are the two most prevalent bone diseases, having considerable morbidity and being responsible for very high healthcare costs. Furthermore, the progressive aging of the population has resulted in a notable increase in their prevalence in the general population[1].

The possible relationship between arthrosis and osteoporosis has been the object of intense debate in the last four decades. In 1972, Foss and Byers[2] indicated that patients with hip fracture rarely had coxarthrosis. Subsequently, numerous studies have been published, often with contradictory results, as well as editorials and bibliographical reviews which have tried to give a clarifying perspective according to the evidence available at that the time. However, the different studies differ substantially in many methodological aspects, which makes their comparison, as well as the development of a synthesis of their results, difficult. In fact, at present the question posed does not appear to have singe answer.

For all these reasons, we are going to approach this problem from a temporal perspective, analysing the studies carried out in three consecutive periods. First, we consider the period between 1972 and 1996. Second, the period from 1996 to 2006; and finally, from 2006 to the present.

Period 1972-1996

In a review published in 1996[3] 36 works carried out between 1972 and 1996 were analysed. These studies were mostly of a transverse design, and gave rise to two observations. One of these was the general clinical impression that no signs of arthrosis were found in the femoral necks extracted during surgery for hip fracture, and furthermore, that this type of fracture was infrequent in patients with coxarthrosis. The second observation was in the phenotype differences which are usually seen in patients with arthrosis or osteoporosis. So, while those patients with arthrosis tended to have an endomorphic biotype, those with osteoporosis usually have an ectomorphic biotype.

In terms of the primary objectives which were addressed in this period, most of the studies were aimed at examining the affect of arthrosis on bone mineral density (BMD). However, there was no uniform criterion for the evaluation of the characteristics of both osteoporosis and arthrosis. For example, on occasion, arthrosis was evaluated through a histological study of the femoral head obtained during surgery for hip fracture. At other times, it was evaluated by means of radiological imaging, essentially using the Kellgren-Lawrence scale, although occasionally other scales were also used, such as the Empire Rheumatism Council Criteria[4]. Finally, in some studies arthrosis was evaluated by the patients themselves using a structured survey ("self-reported osteoarthritis")[5]. In terms of osteoporosis, the measurements of bone mineral density are carried out with different procedures: radiological evaluation of the trabeculae of the proximal femur according to the Singh criteria[4], simple photonic absorptiometry[6], dual photonic absorptiometry[7], quantitative CAT[8] and histomorphometry[9].

The great majority of the studies, as we indicated earlier, were of a transverse design and, especially the older ones, do not include a statistical analysis which permits adjustment for possible confusion factors. In any case, in the majority of the works a significant increase in bone mass was seen in patients with arthrosis, which reached 4-10% in the spine and a little less (3-5%) in the apendicular skeleton. On the other hand, in those isolated cases in which both processes (coxarthrosis and hip fracture) coexisted, the age at which the fractures appeared was higher than that observed in the general population, which supported the idea that arthrosis may exert a protective effect against hip fracture[10]. Lastly, some studies put special emphasis on anthropometric and clinical differences which are observed in the two populations. The osteoporotic patient is usually thin, has a low body mass index and has a greater propensity to fractures, while the arthrosic patient could be, rather, a patient who is overweight, with an increased BMD and muscular strength, and a lower number of fractures[11]. Therefore, according to these first results, the existence of an inverse relationship between osteoporosis and arthrosis seems to have been confirmed. In fact, during this period it was suggested that the latter disease, or a factor related to it, could exert a protective effect against osteoporosis in general, as well as against hip fracture in particular.

Period 1996-2006

Three facts characterise this second period: an advance in laboratory techniques, the establishment of dual energy X-ray densitometry (DXA) as the gold standard for the evaluation of bone, and the carrying out of studies on the arthrosis/osteoporosis relationship with designs which produced more substantial scientific evidence.

Intuitively, the relationship between arthrosis and osteoporosis was shown to be much more complex than had been supposed until then, and new lines of investigation, such as for example, the study of genes involved in both diseases, or the possible beneficial role of antiresorptive drugs on arthrosis. On the other hand, in various studies the existence of an unexpected association was observed between arthrosis of the hands and osteoporosis, as well as between spondyloarthrosis with vertebral fracture. Finally. in the light of new histochemical and imaging techniques (especially DXA), the results of the initial studies on load-bearing joints (hip and knee) have been reconsidered, which has resulted in the widespread opinion that arthrosis and osteoporosis were mutually exclusive diseases.

Bone mass and arthrosis

In the Rotterdam cohort it was observed that, in spite of the fact that those patients with coxarthrosis had higher bone mineral density in the hip than people without arthrosis, the subsequent loss of bone mass, specifically in the two years follo-

wing the fracture, was greater in the arthrosic patients[12]. Furthermore, the loss of bone bore no relation to age, nor to the degree of incapacity of these patients.

On the other hand, Arden et al.[13] also observed that those patients with arthrosis in the hip had a higher BMD than the controls without arthrosis. However, after seven years of follow up the incidence of vertebral and non-vertebral fractures was similar on both groups.

Markers for bone turnover

In general terms, most of the studies carried out during these years showed that patients with arthrosis had increases in markers for resorption. Thus, Naitou et al.[14] confirmed that women with gonarthrosis and with generalised arthrosis had a urinary secretion of pyridinoline and deoxypyridinoline higher than in that in healthy women. Similarly, in another longitudinal study[15] it was demonstrated that postmenopausal women with gonarthrosis showed an increase in the urinary secretion of amino-terminal (NTX) and carboxy-terminal (CTX) telopeptides of collagen type 1, which were the same as those observed in women of the same age with osteoporosis. In terms of markers for formation, there is only one work published in this period[16], in which it was observed that the values for blood osteocalcin were lower in postmenopausal women diagnosed with arthrosis of the hands or knees than in healthy women of the same age.

Antiresorptive drugs and subchondral bone

In spite of arthrosis having been considered classically as a disease of cartilage, it is also possible that the subchondral bone plays some role in the initiation and progression of this disease[17]. On the one hand, the rigidity of subchondral bone appears to favour lesion of the joint cartilage, and on the other, once started, the damage to the cartilage would contribute to the progression of the arthrosis. However, at the end of the Nineties it was confirmed that the subchondral bone of the arthrosic joints had lower mineral content. This phenomenon was associated with an increase in bone turnover[15,18], which led to the suggestion that antiresorptive drugs may be of use in the treatment of arthrosis[19], although this suggestion was not shared by all authors[20]. In any case, in spite of the fact that in some studies favourable results were reported in patients treated with risedronate[21], alendronate or estrogens[22], these results were not able to be confirmed in other studies[23]. In fact, a review published in 2006 on therapeutic strategies for arthrosis[24] it was indicated that more studies would be necessary, perhaps in patients in whom the disease was less advanced, before considering the use of bisphosphonates in the treatment of arthrosis.

Peripheral arthrosis and osteoporosis

We have already said that arthrosis of some load-bearing joints such as the hip or knee is associated with a higher bone mass. In the case of arthrosis of the hands, less information was available, although the few results available suggested a similar association[25].

However, in this second period (1996-2006), some works were published which spoke against this possibility. Thus, in a transverse study it was observed that women with arthrosis in the hands had a lower bone mass in the hip than healthy women[26]. In the same vein, authors observed that erosive arthrosis in the hands was associated with lower T and Z-score indices in the lumbar spine than in controls[27]. Lastly, Haara et al.[28] confirmed that the loss of bone mass over 20 years, evaluated by means of quantitative ultrasound in the calcaneum, was more pronounced in those patients with arthrosis of the interphalangial joints.

Spondyloarthrosis and vertebral fracture

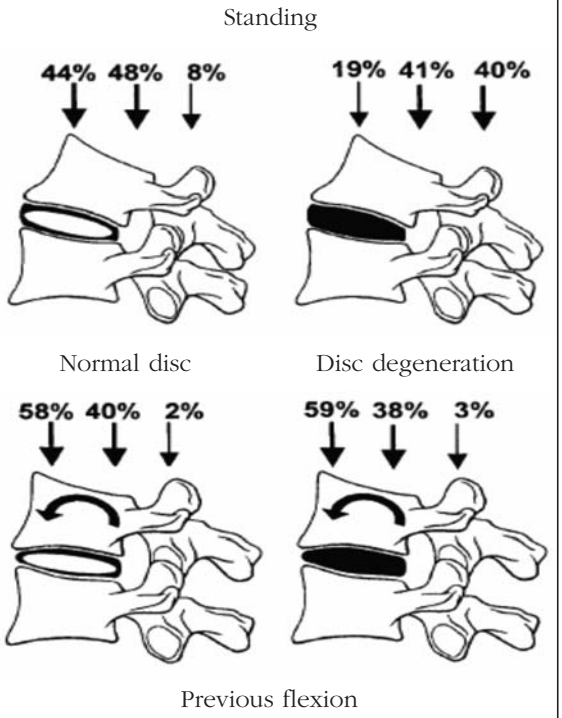
The trabecular bone in the vertebral bodies experiences a series of changes in its architecture (trabecular thinning, loss of interconnections, etc.) which are related to the loss of bone mass which occurs with age, especially in patients with osteoporosis. On the other hand, the vertebral bodies of patients with arthrosis may show other additional changes. For example, the posterior half of the vertebral bodies usually has a higher trabecular density than the anterior[29], which facilitates the appearance of vertebral fractures in those patients with spondyloarthrosis. In turn, these changes may have a relationship with the degeneration of the intervertebral disc. It is well known that the intervertebral discs and the vertebrae interact biologically and mechanically.

In fact, the relationship between the degeneration of the discs and the vertebral bone has been shown in histological and mechanical studies on vertebral segments from cadavers[30-32]. For some authors, the degeneration of the disc would reduce the height of the fibrous ring attracting the neural arches, in such a way that the pressure on the posterior arch would be displaced, improving the resistance to compression of the spinal column. However, the reduction in the mechanical stimulus on the anterior axis of the spinal column would entail in turn a reduction in trabecular volume and an increase in intratrabecular space in the anterior third of the, occasioning a significant reduction in its strength in comparison with the posterior third.

In normal conditions, in a flexing movement anterior to the vertebral column more than 50% of the compressive force is transferred to the anterior half of the vertebral body. In the presence of serious degeneration of the disc, anterior flexing increases the compressive force applied to the anterior zone of the vertebra by up to 300% (Figure 1). To this increased load may be added a reduction in bone mass and the poorer trabecular structure in the anterior third of the vertebra, which could increase the risk of fracture[31].

This sequence of events may explain both the regional variations in BMD of the trabecular bone of the vertebra, as well as the characteristic wedge deformity of the vertebral body in osteoporosis.

Figure 1. Distribution of pressure load in relation to the position of the spinal column and to the degree of degeneration of the intervertebral disc



Pollintine P, et al. Intervertebral disc degeneration can lead to "stress-shielding" of the anterior vertebral body -A cause of osteoporotic vertebral fracture? Spine. 2004;29:774-82.

In line with these findings, Arden et al.[33], in a case and control study, confirmed that the prevalence of vertebral fractures was greater in those patients with thoracic spondyloarthrosis. Subsequently, Sornay-Rendu et al.[34], in a longitudinal study carried out in 634 postmenopausal women from the OFELY cohort, observed that a reduction in intervertebral space is associated with an increased risk of vertebral fracture. The risk was even higher where arthrosis of the thoracic spine was concerned, and was independent of other factors such as age, previous fracture or BMD.

Arthrosis in load-bearing joints (hip, knee) and osteoporosis

In 2003 Glowacki et al.[35] analysed 68 postmenopausal women with advanced coxarthrosis and confirmed that a quarter of them complied with densitometric criteria for osteoporosis. In another study in which 119 patients with advanced arthrosis of the hip or knee participated (83 postmenopausal women and 35 men), it was observed that there was a prevalence of densitometric osteoporosis of around 30% in the women and 20% in the men[36]. Furthermore, over half the women and a third of the men had low bone mass. Therefore, for these authors, their results also contradicted the hypothesis that arthrosis and osteoporosis are two mutually exclusive diseases.

Genetic studies

Taking into account the polygenic hereditary character of both osteoporosis and of arthrosis, the possibility was raised that the two diseases shared some genetic determinants. For example, it has been indicated that certain polymorphisms in the gene for the receptor for vitamin D are associated with a greater degeneration of the intervertebral disc[37], and to an increase in the incidence of arthrosis of the knee, independently of the BMD[38,39]. There has also been an association reported of arthrosis with some allelic variants of the growth factor gene related to insulin type 1 (IGF-1)[40]. Other studies have centred on the possible participation of the Wnt pathway, which, as is known, modulates the differentiation of the pluripotent precursors, allowing their differentiation towards the formation of osteoblasts or to chondrocytes and adipocytes. In any case, the detailed analysis of these aspects exceeds the objectives of this review, so we will not detain ourselves further with it.

Period 2006-2012

Among the most significant aspects of this period, what should first be noted is that a classical line of investigation, the relationship between arthrosis and osteoporosis in the hip, is deepened using the techniques currently available. Similarly to what had occurred previously with the antiresorptives, in this period a series of studies were carried out which analysed the possible beneficial role of strontium ranelate on arthrosis. On the other hand, studies appeared using animal models, in which both diseases were experimentally induced independently, which avoided interference from other confusion factors.

Arthrosis of the hip and osteoporosis

In a study carried out in 2007, Mäkinen et al.[41] investigated the presence of osteoporosis in 61 women with serious coxarthrosis, confirming that nearly a third of them had osteoporosis, while 45% had low bone mass. As might be expected, the osteoporotic women were older and weighed less than those who were not osteoporotic. In addition, they had an increase in markers for formation (PINP and osteocalcin) and resorption (NTx) which were inversely related to bone mass. Therefore, the authors suggested that arthrosis would not be capable of protecting women from suffering osteoporosis. However, in comparing the BMD in both hips in each patient, it was observed that the BMD in the femoral neck of the arthrosic hip was greater than that on the contralateral side, which may explain the lower prevalence of hip fracture which had been reported in patients with coxarthrosis[10].

On the other hand, in a study of cases (562 patients with hip fracture) and controls (803 subjects without that fracture) it was observed that patients with hip fracture had a lower risk of suf-

fering coxarthrosis, which would support the possibility that there might exist an inverse relation between the two processes[42]. However, in this study some confusion variables were not taken into account, such as the body mass index, physical activity, estrogen treatment or the use of bisphosphonate.

In an ultrastructural study carried out using electronic microscopy in 15 postmenopausal women (seven with coxarthrosis and eight with osteoporosis) who were subject to a hip arthroplasty, it was observed that the samples from the patients with arthrosis showed a higher level of new bone formation, the trabecular structure and the collagen fibres remaining intact[43]. On the other hand, in the women with osteoporosis, a marked trabecular reduction and thinning was observed.

Similarly, Marinović et al.[44], after analysing the femoral necks of 24 patients with advanced arthrosis and of 74 patients with hip fracture, observed that the patients with fracture had a wide variation in values of the different histomorphometric parameters related to trabecular bone (bone volume, trabecular separation, trabecular thickness, number of trabeculae), although in most of the cases they would differ from those observed in patients with coxarthrosis.

On the other hand, De Pedro et al.[45] arrived at opposite conclusions. These authors analysed 72 femoral heads obtained after arthroplasty in 56 patients with coxarthrosis and 26 patients who had suffered a hip fracture. The histological study demonstrated the changes which might be expected in both diseases: degenerative changes in the cartilage, cystic cavities and preservation of the thickness of the trabecular tissue in patients with arthrosis, as opposed to less trabecular tissue, thinner trabeculae and less quantity of osteoid tissue in the subjects with hip fracture. However, the histomorphic analysis revealed that more than 40% of those patients with arthrosis had a reduction in trabecular volume, which would support the idea of a coexistence between osteoporosis and arthrosis.

Finally, in recent times, Castaño-Betancourt et al.[46], in a study carried out in patients with atrophic arthrosis characterised by the existence of cartilage degradation without the formation of osteophytes, confirmed that patients with this type of arthrosis had lower BMD and a higher risk of fracture than patients with the usual forms of arthrosis, and than the healthy controls.

Strontium ranelate and arthrosis

Strontium ranelate is a drug used in the treatment of postmenopausal osteoporosis which has shown its efficacy in the reduction of vertebral and non-vertebral fractures[47,48]. Furthermore, in a post-hoc study carried out using grouped data from these studies[49], it was observed that treatment with strontium ranelate over three years would result in a reduction in the radiological progression of arthrosis in the spinal column in women with osteoporosis and spondyloarthrosis.

This effect was observed both in women with a previous vertebral fracture as well as in those with no fractures. Similarly, a recent clinical trial[50], has indicated that treatment with 1 or 2 grams daily of strontium ranelate over 3 years reduces the radiological progression of arthrosis in the knee, evaluated by the height of the internal femorotibial compartment. In addition, with a dose of 2 grams daily a greater improvement in symptoms was observed.

Studies in animal models

In 2007, Calvo et al.[51] published the results of an experimental study which tried to analyse the effect of osteoporosis and arthrosis in experimental animals. Arthrosis was induced in the knees of female rabbits by means of a section of the anterior cruciate ligament and partial meniscectomy, while the osteoporosis was provoked through bilateral oophorectomy and the administration of glucocorticoids. These authors observed that the osteoporosis increased the seriousness of the changes in the cartilage of the knee. Furthermore, the cartilage lesions were negatively correlated with the lumbar BMD, and with the subchondral bone, although in the latter, without being statistically significant. As a result, they suggest that, at least in this animal model, there would be a direct association between the two entities, although it is not known if the acceleration of the arthrosis induced by the osteoporosis is the result of an estrogen insufficiency, an alteration in the biomechanics of the subchondral bone, or of both at the same time.

For their part, Bellido et al.[52], using a similar model, arrived at the conclusion that the increase in resorption which is observed in the subchondral bone of the animals with osteoporosis would alter its quality, which would aggravate the damage in the cartilage of the joint.

Conclusions

In spite of the fact that for decades it was considered that osteoporosis and arthrosis were mutually exclusive diseases, in the last few years the possibility has been indicated that both processes coexist, and that they even bear a direct relationship on each other. However, the analysis of the possible relationship between the two processes is problematic due to various factors, some of which have been suggested in different studies[17,28,35,53,54]. Firstly, it is possible that there are different patterns of association as a function of the location of the arthrosis. Thus, when the arthrosis affects load-bearing joints, essentially the hip and the knee, a higher BMD is usually found, which in spite of this does not appear to protect the arthrosic patient against the risk of fracture. Something similar occurs in patients with spondyloarthrosis, which presents a higher risk of vertebral fracture. On the other hand, in some cases of arthrosis in peripheral joints, such as in the hands, it is not unusual to find reduced BMD, locally or overall.

Secondly, along with methodological aspects, the influence of certain confusion variables such as

race, body weight, physical activity or alcohol consumption should be taken into account, which may explain, in themselves, the inverse relationship which has been reported between the two entities.

Thirdly, on occasion there may be certain circumstances which influence both processes, such as, for example, estrogen deficiency, a well-known risk factor of osteoporosis, which also appears to contribute to the degeneration of articular cartilage in some experimental models, or the pain of arthrosis of the hip, which may diminish physical activity and increase rolling gait, with the consequent reduction in bone mass and a higher propensity to falls.

Lastly, although not the object of this review, the possible interaction between environmental and genetic factors, which may cause different levels of expression of the genetic susceptibility to suffer one or other disease, should be considered.

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