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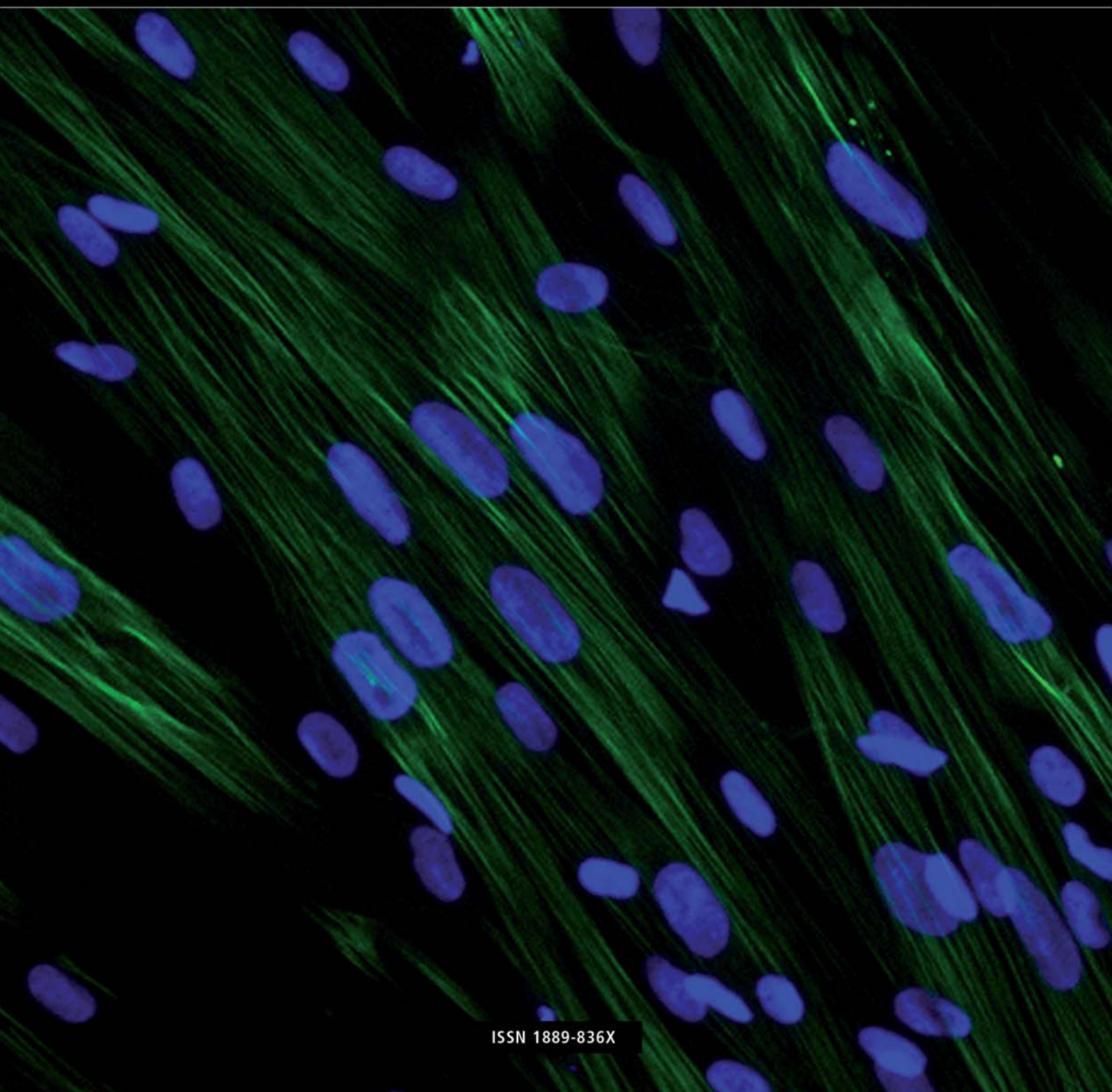

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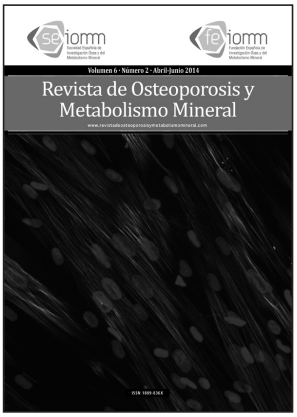

Our cover
Immunolocalization by efflorescence of alpha actin in fibroblasts cultures differentiated myofibroblasts.

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Antonio Casado Díaz, Raquel Santiago Mora y José Manuel Quesada Gómez

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SUMMARY

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Analysis of epigenetic modifications in bone cells: osteoblasts are osteoblasts isolated from bone a good model to study changes in DNA methylation?

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Epigenetics is the study of the mechanisms which regulate gene expression in a stable and hereditary way, but without altering the DNA sequence[1]. This field of research has gained importance in recent years and it is postulated that it may explain the process of differentiation of bone cells, the appearance of bone metabolic diseases, as well the inheritability of certain pathologies (for recent reviews, see[1-3]). Epigenetic mechanisms include post-translational modification in histones, regulation of protein synthesis by means of microRNA and DNA methylation.

Recently, it has been proposed that changes in levels of methylation of genes may alter the differentiation of osteoblasts and osteoclast precursors in bone tissue. For example, the transcription factors osterix and DLX5, estrogen receptor α, as well as osteopontin, are co-regulated through the methylation of DNA[4-6]. Moreover, levels of DNA methylation may be regulated by mechanical stimuli, as is the case with the promoter of osteopontin[6], suggesting that some of the effects of mechanical stimulation are due to the regulation of gene expression by epigenetic mechanisms. It should be mentioned that the methylation of the promoter regions of DNA in certain genes may regulate their expression at different stages of cell differentiation. Such is the case with alkaline phosphatase and sclerostin[7-9]. While the degree of methylation in the promoter region of alkaline phosphatase increases as the osteoblast line cells are differentiated, leading to the silencing of its expression in the osteocytes, the opposite occurs with sclerostin, whose promoter is methylated in the osteoblasts and is demethylated in the osteocytes.

Similarly to osteoblast differentiation, the genes which code for RANKL and OPG are also regulated by their levels of methylation[10], thus affecting the generation of osteoclasts, a process which depends on the relative expression of RANKL and OPG[11]. As occurs with the osteoblast lineage cells, changes in the levels of methylation of DNA also accompany the differentiation of the osteoclast precursors. This results in differences in levels of expression of genes fundamental to osteoclast function such as cathepsin K and tartrate-resistant acid phosphatase[12]. It has also been suggested that aberrant patterns of methylation of DNA may cause pathologies in which there are alteration in bone metabolism. For example, a reduction in levels of methyltransferase DNMT1, an enzyme involved in the maintenance of genome methylation, results in loss of bone mass[13]. Similarly, changes in gene expression in the chondrocytes are associated with changes in the methylation of DNA (for example, in the gene for type x collagen or of various metalloproteins in the matrix[14,15]) could be contributing to the generation of osteoarthritis.

This work is a continuation of earlier studies from the same group in which were demonstrated the role of DNA methylation in the expression of the osteocyte protein sclerostin[9], the marker for bone formation alkaline phosphatase[7], and of the cytokines involved in the generation of the RANKL/OPG osteoclasts[10]. In the manuscript of Delgado-Calle et al.[16] they explore and compare the presence of methylated CpG in purified DNA from human bone and from primary osteoblast cultures obtained also from patients with osteoporotic fractures or arthritis. The authors analysed the levels of methylation in the bone and in the

cultured osteoblasts and found a similar pattern in terms of the average level of methylation, both if all the loci were analysed or only those related to bone. Consistently, a fraction of the genes analysed deviated from the general relationship. In particular, the list of genes related with bone metabolism and which are found to be differentially methylated in preparations of bone and cultivated cells includes the receptor for parathyroid hormone, members of the Wnt and TGFβ pathway stimulation chain, and interleukins and chemokines. Modifications in the expression of these genes could have profound effects on the maturation, proliferation and survival of bone cells. However, the composition of the cells present in the bone, and the fact that the cells have been cultivated in one or two passages, should be taken into account. In particular, the majority of the cells in bone are osteocytes, not osteoblasts. Furthermore, the presence of bone marrow cells in the fragments of bone may also confuse the results. Another factor which may explain the differences found is the fact that the osteoblast cells were exposed to an artificial medium, and in an incubator. Although promising, the result reported in this work need to be complemented by more detailed studies, separating the osteoblasts from the osteocytes to evaluate the contribution of each cell population in the methylated loci.

In summary, the work of Delgado-Calle et al. demonstrates that the population of methylated genes in bone cells varies depending on the source of the material. The conclusions of the study should be treated with caution due to the difference in the types of cells present in the bone, compared with primary cultures, and the small number of replicas. However, it shows the importance of corroborating the results obtained in cell cultures with animal studies or with human samples.

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Comparative epigenomic analysis of bone tissue and primary osteoblasts

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Summary
Objectives: Epigenetic mechanisms, and in particular cytosine methylation in the promoter regions, modulate the expression of many genes. However, their role in skeletal homeostasis has scarcely been studied. In particular, it is not known if the patterns of methylation of bone cells in culture are a good reflection of that which occurrs in bone tissues. The aim of this work was to explore the possible differences in cytosine methylation in human bone and in osteoblasts.
Material and methods: To achieve this we carried out a genome-wide study, analysing the degree of methylation of 23,667 *loci* and comparing the results in samples of bone tissue and in cultures of primary osteoblasts.
Results: Overall, we observed a good correlation between the two sample types, both in the whole group of *loci* (r^2=0,87; $p<10^{-50}$), and in those located in genes involved in bone metabolism. However, some of the *loci* (7-8%) deviated from this general tendency and showed differences in methylation greater than 20%.
Conclusions: These results indicate that the methylation data obtained in cultures are not necessarily a true reflection of that which occurs in tissues, which means that care should be taken when extrapolating such results to an *in vivo* situation.

Key words: *DNA methylation, epigenetics, osteoblasts.*

Introduction

Some common skeletal diseases, such as osteoporosis or arthrosis, have a clear tendency to familial aggregation, which suggest that their hereditary component is significant[1]. In fact, in various studies it has been estimated that heredity explains up to 50-80% of the variability in bone mass[2,3]. However, the allelic variants identified in studies of candidate genes and genome-wide association studies (GWAS) explain barely a small proportion of this hereditary component[4-6]. Epigenetic mechanisms may contribute to the explanation of this phenomenon. These mechanisms permit the adaptation of the expression of genes to environmental conditions. This includes DNA methylation, posttranslational modifications of the histones, the non-coding RNA and the general structure of the chromatin[7-9].

In human DNA, most of the cytosines which are followed by a guanine are methylated. It is thought that this gives stability to the DNA. However, in the promoter regions of many genes there are zones rich in cytosines followed by guanine (called CpG islands) which may be methylated or not[10]. The degree of methylation of these islands is correlated with transcriptional activity: in general, the greater the methylation, the lesser the gene expression[11,12].

There are scarcely any studies of CpG island methylation in bone or in osteoblasts, especially in humans. Nor is it known whether or not the patterns of methylation in CpG islands in the osteoblasts are comparable with those observed in bone. Therefore, the objective of this work was to explore the methylation of cytosines throughout the whole of DNA in samples of human bone, and to compare those results with the patterns of methylation in primary osteoblasts in culture.

Material and methods

Bone and osteoblast cultures

Samples were taken of trabecular bone in the femoral head of women undergoing hip arthroplasty (fractures, arthrosis), using a serrated trocar. The cylinders were obtained from the central region of the head, avoiding the subchondral bone and the areas of fracture and osteotomy, as has previously been described[13]. After extensive washing in PBS the samples were frozen in liquid nitrogen or placed in plastic flasks in Dulbecco's medium supplemented with 10% bovine serum and antibiotics to obtain the osteoblasts from the explants[14].

Analysis of the methylation

After pulverising the bone fragments the DNA was isolated by a procedure previously published[12]. A similar procedure was used to extract the DNA from the confluent osteoblast cultures, from first or second passes[15]. To analyse the methylation, methylation arrays were used (Infinium Human Methylation 27 DNA bead-chip analysis, Illumina) which examined around 27,000 CpG loci located in the promoter regions of some 14,500 genes. The degree of methylation of each locus is expressed as a value of β, which varies between 0 and 1 and is proportional to the methylation (0-100%). The details of the method have been published previously[16].

Analysis of the results

The values of β were multiplied by 100 in order to estimate the percentage of methylation. The average values methylation observed in 15 bones from patients with fracture and in 15 from patients with arthrosis, and who were included in an earlier study[16], were calculated. The average age was 77 years. The results were compared with the average methylation observed in two osteoblast cultures (one from a bone with fracture and the other with arthrosis), which, to reduce sources of variability, were analysed together in the same arrays as the bone samples. To compare the methylation in the two types of sample correlation and linear regression tests were used. Bioinformatic databases and relevant literature were searched in order to identify the genes related to bone.

Results

A total of 23,667 loci were explored. As is shown in Figure 1, when all the CpG loci explored were analysed together a direct correlation was found between the levels of methylation in bone and in the osteoblasts (r^2=0.88; $p<10^{-50}$). Also, in general terms, the average methylation in both types of sample was similar (slope of the regression line b=1.009; intercept -4). However, there was a significant number of loci which deviated from this relationship (Figure 1). To analyse whether these deviations depended on genes not related to bone a limited sub-analysis was carried out of 658 loci located in 319 genes which were clearly related to skeletal homeostasis. The result was similar to that in the overall analysis (Figure 2). There was a general correlation between the levels of methylation in the two samples (r^2=0.87; $p<10^{-50}$), but a significant proportion of the genes deviated from the general relationship.

Restricting the analysis to the 319 bone genes (in which 658 loci were explored), the methylation in bone was slightly higher than in the culture (average difference 3.8%; $p=2.4 \times 10^{-15}$; Figure 3). Specifically, of the 658 loci, 117 (17.8%) showed differences greater than 10%. Of these, 61% were more methylated in the bone tissue than in the culture, while in 39% of the loci the methylation was greater in the cultures. In 45 loci the difference in percentage methylation was greater than 20 points, the excess methylation being equally distributed, in this case, between the bone tissue and the cultures. The genes in which these loci were situated are shown in Table 1.

Discussion

The analysis of the epigenome, and in particular the pattern of DNA methylation, is a subject of growing interest, given the role which it plays in determining the pattern of gene expression across the different stages of differentiation of the cell lines, as well as in their adaptation to changing environmental conditions. Its role in some disea-

ses also appears to be important, especially in neoplastic processes[17]. In fact different studies have related the changes in the methylation of the promoters with alterations in the expression of genes facilitating or inhibiting the development of tumours[18-20]. However, little is known about the role of patterns of methylation in non-tumorous diseases of the skeleton.

One of the factors which makes the analysis of the epigenome difficult is that, differently from the genome, the epigenome is specific to each tissue. This is logical, given that the patterns of gene expression need to be aligned with the specific functions of the tissue (in fact, with those of each type of cell). Hence, given difficulties in obtaining samples of the skeleton, there is little information on the epigenome of bone.

Our group has recently published an analysis of the pattern of methylation in bone tissue in patients with osteoporosis and with arthrosis[16]. In this study we have used these data to compare them with the patterns of methylation in primary osteoblasts in culture, with the aim of determining the extent to which they are similar. This analysis is important in exploring whether or not cells in culture are a good reflection of the pattern in tissue and, as a consequence, if the changes induced by various manipulations of the cultures may be relevant to tissue. In this whole genome study, in which we analysed some 23,000 loci, we confirmed that, in general, there is a good correlation between patterns of methylation in bone and in primary osteoblasts in culture. However, some genes clearly deviate from this pattern. The deviation does not follow a systematic pattern, and affects both genes which have been related to bone metabolism as well as others. Overall, 17-18% of the loci (located in genes related or not to bone metabolic pathways) had deviations in the degree of methylation of greater than 10%. The proportion of genes with differences higher than 20%, certainly significant from a biological point of view, was 7-8%, similar in the loci as a whole and in those located in the sub-group of genes related to bone. There are various reasons which may explain these differences. On the one hand, the culture itself may induce phenotypical changes in the cells, including changes in the patterns of expression and gene methylation. On the other, in bone tissue there are various cell lines, not only osteoblasts, which are not represented in the cultures. Unfortunately, it is not possible to cultivate osteocytes, a type which is highly abundant in bone, to carry out a comparative study similar to that carried out with osteoblasts.

In conclusion, the results of our study indicate that there is a good overall correlation in patterns of methylation between bone tissue and osteoblasts. However, some genes have clearly divergent patterns, with a similar frequency in the subgroup of genes related to bone metabolism to that in the genes analysed in general. Therefore, methylation data observed in culture may not be representative of the situation *in vivo*.

Figure 1. Percentages of methylation in bone tissue and in osteoblast culture across all loci analysed

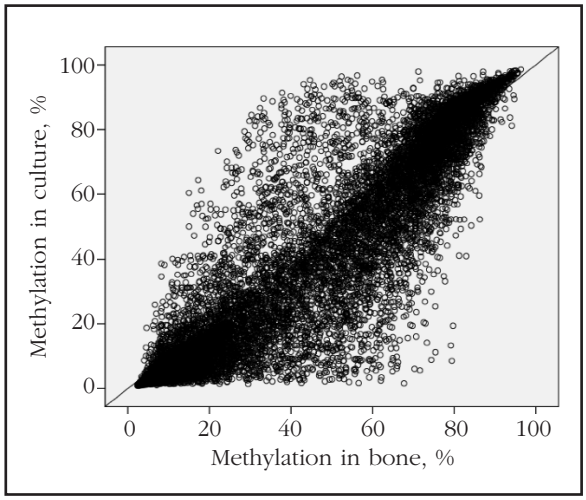

Figure 2. Percentages of methylation in bone tissue and in osteoblasts cultures in loci corresponding to genes related with bone metabolism

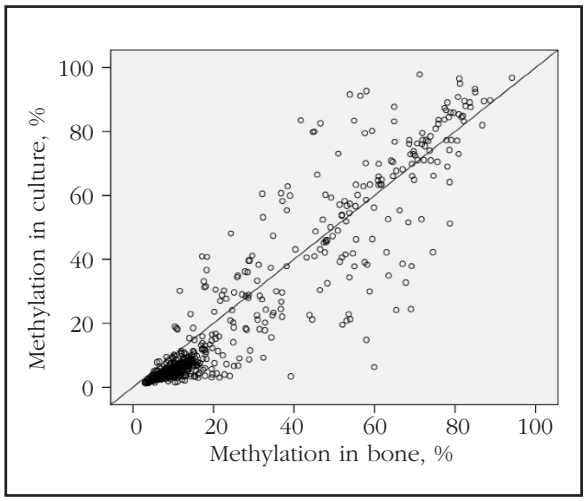

Figure 3. Distribution of frequencies of the differences in methylation between bone tissue and osteoblasts in culture. Only the data corresponding to the genes relating to bone metabolism are shown

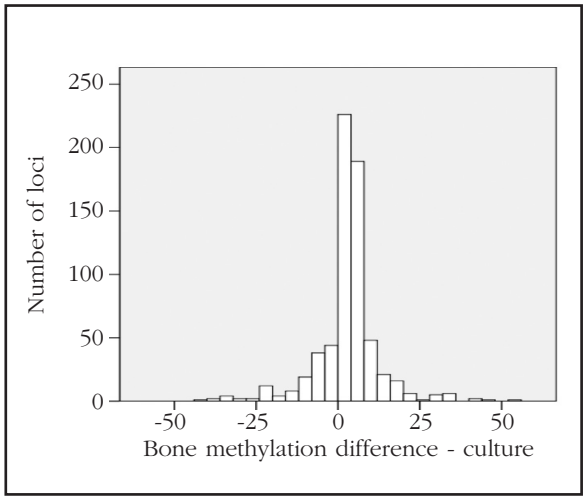

Table 1. Genes related to bone metabolism in which were observed differences in methylation between bone tissue and primary osteoblast cultures greater than 20 percentage points. The number of CpG loci with differences in methylation is indicated

Gen	Nº loci
ACVRL1	1
AMH	1
APC	1
AR	2
ATP6V0D2	1
BGN	1
CDKN2B	4
CHRD	1
COL3A1	1
CXCL12	1
DLX5	1
ENG	1
FGF1	1
FGFR1	2
FKBP1B	1
GDF5	1
IL1B	1
IL1RN	1
ITGAM	1
LGALS1	1
MAP4K1	1
MAPK1	1
MAPK10	1
MSX1	7
NR3C1	1
PTHLH	1
PTHR1	1
SFRP1	1
SMAD2	1
TGFB3	1
TNF	1
TRAF1	1
WISP1	1
WNT6	1

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Risk of major osteoporotic or hip fracture in patients with cerebrovascular accident in the acute phase. Multicentre prospective study

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Summary

Objetives: Hemiplegic patients are considered to be a population at risk of suffering osteoporotic fractures. The aim of this work is to understand the absolute risk of fragility fracture in patients with cerebrovascular accident (CVA) and the osteometabolic state of patients with ictus in the acute phase, as well as confirming if there are baseline differences compared to a control group without cerebrovascular pathology.

Patients and method: Multicentre prospective study carried out in five Spanish hospitals. Two groups were established: a) patients with ictus of less than three months development, and b) a control group from a population without cerebrovascular disease. History of fragility fractures, number of falls in the previous year, bone mineral density (BMD) in the hip, FRAX® index, determinations of biochemistry and bone markers - calcium, phosphorus, alkaline phosphatase, vitamin D, parathormone (PTH), and carboxy-terminal telopeptide of collagen type I (CTX) - were analysed.

Results: A total of 82 patients were studied: 50 patients with CVA and 32 controls. 12% of those patients with CVA had an increased risk of suffering a hip fracture, and 8% an increased risk of a major osteoporotic fracture. In the control group the risk was greater. The hemiplegic patients had BMD in the hip lower than those in the control group, although the differences in both variables were not statistically significant.

The levels of CTX were higher in patients with CVA, this being the sole determination which showed a statistical difference between the two groups studied.

Conclusions: The patients with CVA had values of markers for bone resorption (CTX) significantly higher and a BMD in the hip lower than those in the control group.

Key words: *cerebrovascular accident, BMD, fragility fracture.*

Introduction

As is universally accepted, the significance of osteoporosis is rooted in the risk of it provoking fractures. Specifically, it is hip fractures which have greatest significance given their functional and economic repercussions and their impact on mortality rate. Hip fractures are considered to be a multifactorial occurrence, making the study and prioritization of its various factors highly important.

The importance of stroke in the risk of hip fracture started to be raised in 1997, after a study carried out in the Japanese population by Suzuki at al.[1] Subsequent studies[2-5] have reinforced these findings, leading to the consideration of hemiplegic patients as a population at risk, and recommendations for the systematic determination of bone mineral density (BMD) and the use of bisphosphonates during the rehabilitation period[6].

There are various etiopathogenic theories which have tried to explain this outcome, varying from an increase in falls, the consequence of a change in gait[1], to an accelerated reduction in bone mass provoked by immobility[7]. This suggestion has been reinforced by some studies which found significant differences in bone mass between the paretic side and the healthy side, as well as relationships between levels of BMD and residual functional activity[7-9].

Some authors have suggested that the increased risk of fracture is rooted in the vitamin D deficit[10] which patients with hemiplegia usually suffer, a deficit attributable to nutritional deficiencies and low exposure to sun. The impact on bone quality and a greater risk of falls due to muscle weakness could explain the increase in fractures[11].

Genetic alterations have also been blamed, since there may be a greater presence in stroke patients of a polymorphism in the OPG-118c/C gene, which controls the synthesis of osteoprotegerin, although this alteration has only been associated with stroke with hemorrhagic etiology[12].

More recent theories suggest an alteration in bone remodelling, much increased during the first year post-stroke, as the cause of the deterioration in bone quality[13-15].

But it is also possible that those patients who suffer a cerebrovascular accident (CVA) arrive at this outcome with lower levels of BMD and therefore a greater risk of suffering osteoporotic fractures[16].

The explanation of this situation may be found in the relationship, not yet sufficiently clarified, between dyslipemia, arteriosclerosis and osteoporosis, with possible common etiopathogenic mechanisms[17].

The aim of this study was to discover the absolute risk of major osteoporotic and hip fractures using the FRAX® tool in patients with CVA. Our secondary objectives were to try to evaluate the existence of baseline differences in the osteometabolic parameters and BMD between patients with stroke and a control group without cerebrovascular pathology.

Patients and method

A comparative, multicentred, prospective study, was carried out in 50 patients with CVA and 32 controls, due to non-1:1 pairing, with the participation of the rehabilitation services of five Spanish hospitals. The inclusion of patients started in March 2011, ending in June 2013. The study was authorised by the ethics committees for scientific research of the participating hospitals. Due to budget restraints it was not possible to carry out the control group densitometry studies in one centre. All the patients, both in the study group and in the control group, gave their informed consent.

Inclusion and exclusion criteria

a) Group of patients with CVA: the inclusion criteria were:

- Patients referred to the rehabilitation clinic with a diagnosis of CVA of at least 3 months development, whether there was etiology ischemic or hemorrhagic.
- Aged between 60 and 80.

The following were established as exclusion criteria:

- Patients who, prior to the stroke, were bedridden, due to any pathology, for more than 24 weeks.
- Patients who were non-ambulant prior to the stroke.
- Previous CVA with functional impacts.
- Patients diagnosed with secondary osteoporosis due to hiper- or hypoparathyroidism, hipo- or hyperthyroidism, hypogonadism in males, treatment with oral corticoids for more than 3 months, chronic alcoholism with the presence or hepatic alterations.

b) Control group:

This was made up of patients who attended the rehabilitation clinic for any type of pathology but who did not suffer from a vascular disease (CVA, ischemic cardiac pathology, arterial ischemia in the lower limbs).

The selection was made according to a system which paired age and sex.

The other exclusion criteria were the same as with the CVA group: being bedridden, non-ambulant, secondary osteoporosis.

Variables studied in the CVA group

- Age and sex.
- Ischemic or hemorrhagic CVA etiology.
- Ability to ambulate according to the functional ambulation classification of the of the Hospital of Sagunto (FACHS) which stratifies this activity in the following way:

Level 0: Incapable of, or zero, ambulation.

Level 1: Non-functional ambulation.

Level 2: Ambulation only inside buildings and the home.

Level 3: Ambulation in the surroundings of the home with a perimeter of less than 600 metres.

Level 4: Independent ambulation in the community but with abnormal gait (any type of limp).

Level 5: Normal ambulation without lameness or any limitations.

- History of fragility fractures in the 10 years prior to CVA, and location.

- Number of falls in the year before the stroke.
- BMD in the hip.
- T-score: considered to be osteopenia when between -1 and -2.5, and osteoporosis when T< -2.5.
- FRAX® index: there is considered to be a risk of fracture when the percentage values for major osteoporotic fracture is ≥10 and for hip fracture, ≥3.
- Analytical tests:
* Biochemistry: glucose, cholesterol and triglyceride.
* Bone markers: total alkaline phosphatase, 25-hydroxyvitamin D, PTH, calcium, phosphorus and carboxy-terminal telopeptide of collagen I (CTX) in blood.

In the control group the same data were collected, with the exception of those related to the hemiplegia and functional status.

Statistical analysis

An initial statistical analysis was carried out, calculating the frequencies and percentages of the categorical variables. The comparative statistical study was carried out using contingency tables with residual analysis.

For the quantitative variable the mean plus standard deviation were calculated using the t-Student test to perform the comparative analysis. The level of statistical significance was established at $p<0.5$ for all the variables analysed.

Results

A total of 82 patients were studied: 50 patients with CVA and 32 who made up the control group.

With respect to sex, in the CVA group there were 24 men (48%) and 26 women (52%), with no significant difference in that of the control group which was made up of 14 men (43.75%) and 18 women (56.25%).

There were also no significant differences in the average age of the two groups: CVA: 70.32 years (SD±5.8); and control group: 72.44 years (SD±6.6).

Ischemic processes determined the etiology in 39 cases (91%) and cerebral haemorrhage was diagnosed in 4 (9%); no cause was given in 7 patients.

The majority of patients in the study had an ambulant capability of 3 or 4 on the FACHS (CFMHS) scale (Table 1).

The patients with CVA had suffered a greater number of falls in the year before the study; on the other hand the control group had a greater history of fragility fractures (Table 2).

T-score at hip: 66% of stroke patients and 50.15% in the control group had a T-score levels of osteopenia or osteoporosis, with no significant differences between groups (Table 2).

BMD in the hip: The hemiplegic patients had a lower average level of bone mass in the hip than the control group, but the differences were not significant (Table 4).

FRAX® index: In the control group the number of patients with a high risk of suffering a fragility fracture was higher than in the CVA group, although the differences were not statistically significant (Table 2).

Biochemical tests: Levels of cholesterol were higher in the control group, but not glycemia or triglycerides, the differences not being significant in any case (Table 3).

Analytical markers for bone metabolism: Levels of vitamin D: In the CVA group 32 patients (68%) had levels lower than 30 ng/ml, classed as vitamin D insufficiency or deficiency; in the control group 22 patients (71%) were insufficient or deficient in this vitamin. The differences between the groups was not significant.

There were also no significant differences found in the values of calcium, phosphorus and PTH.

The levels of CTX were significantly higher in patients with CVA (Table 4).

Discussion

This study was intended to show the baseline in relation to the risk of osteoporotic fracture of patients who had suffered a recent CVA, on the assumption that most of the findings will be more related to their pathological history and life style, than to the impact which, with the passage of time, a stroke could have on the bone system, with motor repercussions. As might be expected, in the majority of patients the stroke had an ischemic etiology, a rate of 91% being the highest of the rates found in the neurology services[18], although it is possible that the higher mortality of the hemorrhagic stroke results in a greater proportion of ischemic strokes in the rehabilitation services.

To evaluate functional status we used the FACHS (CFMHS)[19], which is a valid scale and widely used in rehabilitation services. We preferred this to other better known scales such as the Barthel Index, because it focuses on the ambulatory capability of the patient, the daily activity most relevant to development of bone health.

In relation to the results obtained with this scale of ambulation, we believe it is important to to highlight the fact that while it may be limited, 62% of stroke patients have autonomous ambulation, a surprising situation in acute phase CVA, although there may be a selection bias due to the difficulties (transport, tests, etc) which those patients with the most sever motor impairments may have.

We have chosen the FRAX® index because, in spite of some of its recognised limitations, it is increasingly being used a tool. A cut off point of 10 was established for major osteoporotic fracture, and 3 for establishment of a high risk of hip fracture, due to these being the minimum cut off values which, when the study started, were recommended and used by other Spanish authors[20,21]. While there is increasing evidence that the FRAX® index underestimates the risk of fractures in the Spanish population, major osteoporotic fractures in particular, although the prediction of hip fracture is more sensitive[22], we should emphasise that this is the index most relevant to our study.

Using these cut off levels, we found that 12% of patients had a high risk of suffering a hip frac-

Table 1. Capacity ambulation following FACHS

	Frequency	Percentage
Level 0: No ambulation	7	15.21%
Level 1: No functional ambulation	3	6.50%
Level 2: Only at home	7	15.21%
Level 3: Independent perimeter less than 600 meters	13	28.26%
Level 4: Separate up to ANOMALOUS	12	26.08%
Level 5: Normal ambulation	4	8.69%
Was not collected	4	8.6%

ture, and 8% of suffering a major osteoporotic fracture in the next 10 years. We found no similar studies in patients with stroke with which we were able to compare our results.

In terms of the values of BMD in the femoral neck, 58% of stroke patients had osteopenia and 8% osteoporosis. A study published by Hye Won[16], carried out in patients with the same characteristics as ours (acute phase CVA), found rates of osteopenia and osteoporosis in the spine of 39% and 43% respectively, although we should remember that, according to studies carried out by Díez Curiel, the reduction of BMD is more common in the spine than in the hip[23].

It may appear surprising that the control group, despite being similar in sex and age to the patients with CVA, have a greater risk of suffering a major osteoporotic or hip fracture, a situation which may be correlated with a greater number of fragility fractures in the 10 years prior to the study. But this finding could make one suspect selection bias and therefore a limitation to our results, which has favoured the inclusion of patients with osteoporosis in the control group. We should not forget the fact that the prevalence of osteoporosis in a general rehabilitation clinic is very high, as Serralta[24] reported, finding this pathology in 53% of his patients.

The relationship between dyslipemia and bone metabolism has been described by various authors, but without conclusive findings[17]. In our study we found no significant differences from the control group in any of the biochemical parameters. In absolute terms, the rates of cholesterol were lowest in the patients with CVA. These results need to be treated with caution since we did not record possible lipid-regulating treatments, which is a limitation of the study, especially given the significant effects the statins appear to have on bone metabolism and in the reduction in risk of fractures[17].

What is notable is the significant number of patients with vitamin D insufficiency or deficiency in both groups, a finding which points once again to prevalence of the deficiency in this hormonal system in Spain, which is found in 30% of the general population, reaching to 87% in institutionalised older people[25,26].

The most significant of the analytical findings is the increase in the marker for bone resorption CTX in the group with stroke. This finding has been reported in other studies which, like ours, have been carried out in acute phase stroke patients[15] an which correlates with a greater loss of bone mass in the hip[13].

For these authors there is a correlation between high levels of CTX and the degree of motor impairment; in this case mechanostasis would be the most influential factor in the increase in bone resorption in these patients. However, bone metabolism is a complex process in which local and endocrine-metabolic factors are involved with a possible final effect on RANK-RANKL-OPG, which would be the final agent of the bone remodelling process[27].

It is possible that, similar to changes in this system which have been described in Paget's disease, prostate cancer or osteoid arthritis, among others[27], some specific changes in these cytosines may be found in patients with CVA.

In conclusion, if we want to reduce the risk of patients with CVA suffering a hip fracture in the years following a stroke, a programme of physical therapy with load and ambulation should be planned to favour mechanostatic factors, without forgetting the need for antiresorptive treatment, at least for patients in whom a risk of fracture is detected.

Conflict of interest: The authors declare that there are no conflicts of interest.

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Table 2. Frequency and percentage of falls during a year, fractures in the previous 10 years, of patients with osteopenia and osteoporosis in the hip (T-score) and patients with high risk of major osteoporotic fracture (MO) and of hip fracture (HF) in both groups studied

		Fallen	Fractures	T-score: -1 a -2.5	T-score: ≥-2.5	FRAX® MO ≥10	FRAX® HF ≥3
CVA	Frequency Percentage	4 8%	3 6%	29 58%	4 8%	4 8%	6 12.5%
Control	Frequency Percentage	0	8 25%	13 34%	5 15%	4 13%	8 25.8%

Table 3. Biochemical determinations. Averages

	CVA	Control	p value
Glycemia (mg/dl)	108.97	99.68	NS
Cholesterol (mg/dl)	144.97	199.48	NS
Triglycerides (mg/dl)	129.7	114.33	NS

Table 4. Bone mineral density (BMD) of the hip bone and biochemical parameters

	CVA mean±SD	Average control±SD	p value
BMD hip (g/cm²)	0.7216±0.185	0.7609±0.16	NS
Calcium (mg/dl)	9.37±0.46	9.42±0.58	NS
Phosphorus (mg/dl)	3.54±0.70	3.37±0.56	NS
Total alkaline phosphatase (UI/l)	125.32±67	111.97±52	NS
Vitamin D (ng/l)	25.31±11	24.69±11	NS
PTH (pg/ml)	48.73±35	59.99±36	NS
CTX (ng/ml)	0.4362±0.27	0.2907±0.11	0.011

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Comparison of the osteogenic actions of parathyroid hormone-related protein (PTHrP) in diabetic and insulin-like growth factor-I (IGF-I) deficient mouse models

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Summary

Diabetes mellitus (DM) is a metabolic pathology characterised by chronic hyperglycemia due to a deficit in the production and/or action of insulin. DM, above all type I, is commonly associated with osteopenia/osteoporosis and with an increased risk of fractures. Insulin-like growth factor-I (IGF-I), a factor abundant in the bone matrix which plays a significant role in the development and maintenance of bone mass, diminishes with DM. Parathyroid hormone-related protein (PTHrP), a modulator of growth and osteoblast function, acts on osteoprogenitors, promoting osteoblast differentiation and bone regeneration. Its expression is reduced in the presence of DM. In this work we have evaluated and compared the osteogenic actions of PTHrP in mouse models with type 1 DM and IGF-I deficiency. Diabetic mice by injection of streptozotocin had a reduction in bone mass in the long bones associated with an increase in oxidised proteins and a reduction in the expression of genes related to the Wnt pathway and of β-catenin protein, as well as alterations in vertebral trabecular bone. In the mouse model with IGF-I deficit our results indicate the presence of osteopenia both in the femur (associated with an inhibition of the Wnt pathway) and the spine (L1-L5). Our findings demonstrate that the administration of PTHrP, predominantly through its N-terminal domain, modulates the canonical Wnt pathway in relation to its osteogenic actions in a diabetic situation and also, in part, in the absence of IGF-I.

Key words: *PTHrP, diabetes mellitus, IGF-I, osteopenia, Wnt pathway.*

Introduction

Diabetes mellitus (DM) is a metabolic pathology characterised by chronic hyperglycemia due to a deficit in the production and/or action of insulin, responsible for the dysfunction of organs such as the retina, the kidneys, the nervous system and the cardiovascular system[1]. Furthermore, DM is commonly associated with osteopenia/osteoporosis and with an increase in the risk of fractures, due to mechanisms only partially described[2]. DM type 1 (DM1), or insulin-dependent diabetes, is characterised by low levels of insulin and of growth factor similar to insulin type 1 (IGF-I) in the blood and is usually manifested before peak bone mass is reached, while type 2 (DM2) – associated with insulin resistance – is common in adults[3]. Skeletal changes in DM1 include: 1) a reduction in longitudinal bone growth during puberty in adolescents; 2) a reduction in bone mass in the hip, femoral head and spine in adults; 3) an increased risk of fracture; and 4) a reduction in the regenerative capacity of the bone. The characteristics of DM are compatible with a low level of bone remodelling[4-7]. Hyperglycemia induces a lower level of proliferation and function of the osteoblasts. In addition, the products of advanced glycosylation (AGEs) contribute the generation of oxidative stress, increasing bone fragility and the risk of fracture[8,9].

Among endocrine and local factors which have been shown to act on bone, insulin, produced and secreted by the β pancreatic cells and IGF-I, mainly produced in the liver but also in bone where it accumulates in the bone matrix, merit special consideration in osteopathy associated with DM[10,11]. Studies in diabetic type 1 rats indicate the role of insulin deficit in the reduction in the integrity and resistance of bone[12,13]. Furthermore, patients with DM1 have blood levels of IGF-I significantly lower in relation to those found in normal individuals or in patients with DM2[14]. It is known that systemic IGF-I plays an important role in the development and maintenance of bone mass. In fact, mice with an overall deficiency in IGF-I have a size at birth approximately 60% of that of controls, which reduces to 30% at 8 weeks, and have lower levels of bone mineralisation and of bone remodelling[15-17].

On the other hand, the protein related to parathormone (PTHrP) plays a fundamental role in the development of endochondral bone, delaying the differentiation of the chondrocyte growth plates, and acting as an important local regulator for bone remodelling in adults[18]. Homozygous Pthrp-/- mice have lethal perinatal chondrodysplasia; while heterozygous Pthrp+/- mice are viable but exhibit a significant reduction in bone mass[19]. PTHrP has a structural similarity to PTH at its N-terminal extreme, but differs completely from this hormone in the rest of its structure. The middle section and the C-terminal of PTHrP contain different singular epitopes associated with auto/paracrine and intracrine effects in different types of cells[20]. As a consequence of its post-transductional signal processing[21], PTHrP may generate different bioactive fragments: 1) an N-terminal 1-36 fragment; 2) one or many fragments from the middle region whose amino acids 88-91 and 102-106 are nuclear/nucleolar localisation domains (NLS); and 3) a C-terminal fragment which contains the sequence 107-111 known as osteostatin. Although a receptor for this C-terminal region of PTHrP has not yet been successfully isolated, it has been shown that it signals in part through the transactivation of receptor 2 of the vascular endothelial growth factor (VEGF) associated with its actions in the osteoblasts[22-24]. Previous studies have shown that PTHrP reverses the deleterious effects of DM1 on the number of osteoforming cells and the osteoblast function in a regenerating mouse tibia[25]. Furthermore, PTHrP is capable of compensating for the reduction in osteoblast differentiation and the inhibition of the signalling by means of Wnt/β-catenin – a key pathway which stimulates bone formation induced by the high levels of glucose in osteoblastic cells *in vitro*[24,26,27].

Taking into account these considerations, in this work we have evaluated and compared the consequences of insulin deficit (DM1) and IGF-I on the efficacy of PTHrP in inducing osteogenic actions in the mouse.

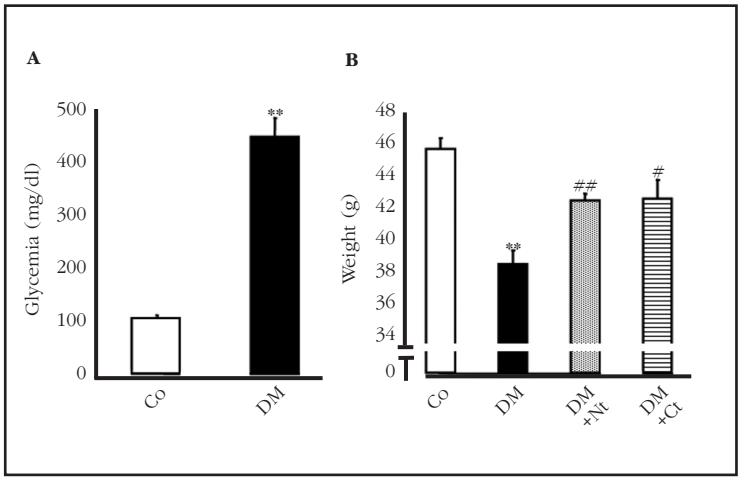
Materials and methods

All the studies carried out in animals were developed with the approval of the committee for experimentation and animal welfare of the Jiménez Díaz IIS-Foundation. The pain and suffering of the animals were palliated in accordance with current European regulations (Directive 2010/63/EU). In addition, the experimental design was adapted to the criteria known as 3R (replace, reduce, refine) to minimise the number of animals which still allow significant results to be obtained[28].

Model of mouse with DM1

Male CD-1 mice of 4 months of age were used (Harlan Interfauna Ibérica, Barcelona), stabilised over two weeks in the vivarium of the Jiménez Díaz IIS-Foundation. The animals had free access to water and a standard diet (8.8 g/kg of calcium and 5.9 g/kg of phosphorous; Panlab, Reus), at 22ºC with cycles of 12 hours of light and 12 hours of dark. To induce DM, the mice were injected intraperitoneally with streptozotocin (STZ) (Sigma-Aldrich, St Louis, Missouri, US), a pancreatic cytotoxin, over 5 consecutive days at a dose of 45 mg/kg body weight in a buffer solution of sodium citrate 50 mM, pH 4.5, or with a saline vehicle (controls)[25]. A week after the last injection blood glucose was measured in blood taken from the mouse tail, using a glucometer (Glucocard G+-meter, Menarini Diagnostics, Florence, Italy), those mice with glycemia ≥250 mg/dl (Figure 1A) were considered to be diabetic. Two weeks after the confirmation of DM, the mice were treated with PTHrP (1-36) (Nt) or PTHrP (107-139) (Ct) (Bachem, Bubendorf, Switzerland), 100 µg/kg in each case, or with phosphate saline buffer, pH 7.4 (PSB) (peptide vehicle) every two days by subcutaneous injection, for a total of 14 days (Figure 1A). 5 mice/group were used in each of these 4 experimental groups.

Figure 1. Description of model of mouse diabetic due to STZ. Shown are the baseline glycemia in control (Co) and diabetic (DM) mice, as well as changes in weight of each of the experimental groups. Nt, PTHrP (1-36); Ct, PTHrP (107-139). The results represent the mean ± SEM of 5 mice/group. **p<0.01 vs Co; #p<0.05; ##p<0.01 vs DM



Two hours after the last injection of each treatment, the animals were weighed and then subsequently sacrificed with a mixture of ketamine (Pfizer, Madrid, Spain) 20 mg/kg and xylacine (Bayer, Kiel, Germany) 5 mg/kg (2:1, v/v). Subsequently the femurs, the tibias (discarding the fibula) and the L1-L5 vertebrae were extracted, with the adjacent muscle eliminated. The long bones were used to obtain cultures of bone marrow-derived mesenchymal cells (BMMCs), or stored (in liquid N_2) for subsequent extraction of RNA or the analysis of carbonylated proteins (at -80ºC). The vertebrae were stored at -20ºC until their incorporation into methacrylate for bone histomorphometry.

Model of mouse deficient in IGF-I

The mice with homozygous IGF-I deficiency (Igf1-null), 3 months old and with a mixed genetic background MF1/129sv, were generated after crossing heterozygous mice with a deletion in exon 4 of the Igf1[15]. The mice were genotyped using Southern Blot after the extraction of genome DNA from the tail with REDExtract-N-Amp™Tissue PCR Kit (Sigma-Aldrich) and characterised by functional criteria[29,30].

Four experimental groups were established with 6 mice per group, control and Igf1-null, treated with PTHrP (1036), PTHrP (107-111) or with PSB. The PTHrP peptides (80 µg/kg in each case) or saline vehicle were administered by subcutaneous injection every 48 hours for two weeks. This dose was chosen because similar doses of these peptides induce anabolic or antiresorptive effects, respectively, in rodents[25,29-31]. Two hours after the last injection the mice were sacrificed, as already described. The long bones were used to obtain BMMCs. The spare femurs were stored in liquid N_2 for later extraction of total RNA, and the L1-L5 vertebrae for histomorphometry.

***Ex vivo* culture of BMMCs**

To obtain the BMMCs from the femurs and tibias obtained from both animal models, the epiphysis was perforated parallel to the diaphysis with a surgical needle of 20G thickness. The marrow cavity was perfused with α-MEM culture medium supplemented with 15% foetal bovine serum, 1% penicillin-streptomycin and 2.5 µg/ml fungizone, and the bone marrow obtained. After various washes a homogenous suspension was obtained which was centrifuged at 1,500xg for 5 minutes at a cold temperature. The cell precipitate was resuspended in the aforementioned medium (without fungizone), and the number of viable cells counted (by exclusion with trypan blue) in an automatic cell counter (Countess™, Life Technologies, Paisley, United Kingdom). Subsequently, the cells were seeded at a density of 1-2, $5x10^6/cm^2$ in 6-well plates in a humid atmosphere of 5% CO_2 at 37ºC[25,32]. Osteogenic differentiation medium was added (the aforementioned medium supplemented with 50 µg/ml L-ascorbic acid and 10 nM β-glycerol phosphate) to the culture the third day after seeding. The cells were kept under these conditions for 14-16 days, with half the volume of the conditioned medium replaced every two days. During this period the BMMCs originating from diabetic or Igf1-null mice were treated *in vitro* with the PTHrP peptides (added when the medium was changed).

Bone densitometry

Using double X-ray absorptiometry (DXA) the bone mineral density (BMD; g/cm^2), the bone mineral content (BMC; g) and the % periosteal fat in the total body, the femur, the tibia and spine (vertebrae L1-L5) (regions of interest) of the anaesthetised mice were measured. The DXA was performed using a PIXIMus I instrument (GE Lunar Corp., Madison, Wisconsin, US). The instrument's programme calculates the cited parameters in different regions of the skeleton (excluding the head) with a coefficient of variation of ±2%.

Bone histomorphometry

The samples of the L1-L5 vertebrae were fixed for 24 hours in 70% ethanol and, later, dehydrated in 96% ethanol for two days and then in absolute ethanol for a further two days. Next, the samples were set in polymerised methyl-methacrylate (Merck, Whitehouse Station, New Jersey, US), following a standard protocol[34]. Then, a series of 7 µm sections were made, as close as possible to the sagittal axis of the spine with a Leica RM 2255 microtome, which were deposited on slides pretreated with Haupt's gelatine, covered with a layer of polyethylene and pressed for 20-24 hours at 60ºC. Before staining the samples were deplastici-

sed with methyl-acetate (Merck) for 15-30 minutes, followed by rehydration with ethanol at decreasing concentrations (absolute, 70% and 50%) and washed with distilled water. The von Kossa stain allows the visualisation of mineralised bone coloured black. Staining with Goldner's trichrome colours the cell nuclei blue, the osteoid borders red, and mineralised bone green. After the staining, the samples were dehydrated and mounted with DPX resin (VWR, Louvain, Belgium).

To determine the histomorphometric parameters, a micrometer coupled to a rectangular grid in the eyepiece of a microscope (Olympus BX41, Olympus, Melville, New Jersey, US) was used[32]. The following were determined: the trabecular volume as against the total bone volume (BV/TV); average trabecular thickness (Tb.Th); the number of trabeculae (Tb.N); and the trabecular separation (Tb.S), according to the criteria of the American Society for Bone and Mineral Research[33]. These parameters were evaluated independently by two observers.

Analysis of protein expression by western transference

To extract the total protein from the femur it was homogenised mechanically in a mortar. The proteins were extracted with RIPA buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS)], supplemented with protease inhibitors (Protease inhibitor cocktail P8340, Sigma-Aldrich) and phosphatases (Phosphatase inhibitor cocktail Set II, Calbiochem, La Jolla, California, US). After incubation for 30 minutes at 4ºC the samples were centrifuged at 13,000 rpm for 30 minutes, and the supernatant collected. The concentration of protein was measured using the bicinchoninic acid method (Thermo Fisher Scientific, Rockford, Illinois, US), using a bovine serum albumin curve pattern. In the protein extracts the carbonylated proteins were quantified by the derivatisation of the carbonyl groups with 2,4-dinitrophenylhydrazine (DNP-hydrazine) using the commercial test, OxyBlot protein detection kit (Millipore, Billerica, Massachusetts, US). The stable protein DNP-hydrazone obtained was detected by immunotransfer. To achieve this, the derivatized proteins (20 µg) were separated by electrophoresis in polyacrylamide-SDS gels at 12.5%, and subsequently transferred to difluoro polyvinylidene membranes (Schelider & Schuel, Keene, New Hampshire, US), followed by incubation with a primary polyclonal anti-DNP antibody and with a secondary antibody conjugated to horseradish peroxidase. The resulting bands were visualised using chemoluminescence (ECL Western Blotting Detection Reagents; GE Healthcare, Buckinghamshire, United Kingdom).

For the analysis of the proteins from the BMMCs, the protein extracts (20 µg) were separated in 8% polyacrylamide-SDS gel with 5% β-mercaptoethanol. Next, the samples were transferred to nitrocellulose membranes (Trans-Blot® SD semi-dry transfer cell, Bio-Rad, California, US). Then the membranes were blocked with skimmed milk at 2.5% in a Tri-saline buffer (Tris-HCl 50 mM, pH 7,5, NaCl 150 mM, Tween-20 al 0,1%). Subsequently, these membranes were incubated in the presence of the primary polyclonal antibody corresponding to β-catenin ([1:10000 dilution]; Abcam, Cambridge, United Kingdom) and goat anti-rabbit IgG combined with horseradish peroxidase [(1:10000 dilution); Santa Cruz, California, US]. As a loading control the expression of β-actin [(1:500 dilution); Santa Cruz] was analysed.

Analysis of gene expression using real time quantitative PCR (RT-PCR)

The total RNA was extracted from the homogenised femur (as has already been described) with Trizol (Invitrogen, Groningen, Netherlands) at 4ºC. The reverse transcription of the RNA obtained to cDNA was carried out with 0.5-1.5 µg of RNA with a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, California, US) in a Techgene thermal cycler (Bibby Scientific Ltd., Staffordshire, United Kingdom), according to the following sequential protocol: 10 minutes at 25ºC, 120 minutes at 37ºC and 5 minutes at 85ºC. The real time PCR was carried out with: 1) specific mouse primers for the following genes of the Wnt[34] canonical pathway: Wnt3a, frizzled 2 (Fz2) and proteins related to receptors for low density lipoproteins 5 and 6 (Lrp5 and Lrp6, respectively) (Table 1), and the reaction mixture SYBR Premix Ex-Taq green polymerase (Takara, Otsu, Japan); 2) TaqMan MGB probes (Assay-by-Design™ System, Applied Biosystems) for cyclin D1 (Ccnd1) and connexin 43 (Cx43), and a reaction mixture with Premix Ex-Taq polymerase (Takara) in a ABI PRISM 7500 thermal cycler (Applied Biosystems). In parallel, ribosomal RNA 18s was amplified as normalising gene[25,31].

The dissociation curves verified the obtaining of single amplification products in the cases in which specific primers were used. The levels of expression in each experimental condition relative to the baseline control were calculated as $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = {}_{treatment}\Delta Ct - {}_{baseline}\Delta Ct$), as has been described earlier[27]. All the determinations were carried out in duplicate.

Statistics

The results were expressed as mean ± standard error of the mean (SEM). The comparison between various groups was carried out using the Kruskal-Wallis non-parametric test. The parametric comparison between two groups was carried out with the Student t test, while in those non-parametric comparisons the Mann Whitney test was used. Those differences with a $p<0.05$ were considered significant, The analysis was performed using the computer programme Graphpad InStat (San Diego, California, US).

Results

Osteogenic actions of PTHrP in a model of osteopenia associated with DM1 in mice

The mice, diabetic due to an injection with STZ, showed a significant reduction in body weight with respect to the controls, which was partly

Table 1. Sequence of the specific primers used for the gene amplification by real time PCR

Primer	Sequence Sense 5'-3'	Sequence Anti-sense 5'-3'
Wnt3a	GCACCACCGTCAGCAACAG	GGGTGGCTTTGTCCAGAACA
Fz2	CCGTCTCTGGATCCTCACAT	AGAAGCGCTCATTGCATACC
Lrp5	CAACGTGGACGTGTTTTATTCTTC	CAGCGACTGGTGCTGTAGTCA
Lrp6	AGATCCATCAAGTGGGTTCATGTA	GAAGCGACTTGAGCCATCCA
18s	ATGCTCTTAGCTGAGGTGCCCG	ATTCCTAGCTGCGGTATCCAGG

reversed on treatment with both PTHrP peptides (Figure 1). In these animals, the DM induced a reduction in BMD and BMC, as well as in the percentage of periosteal fat, predominantly in the long bones, alterations which were in part due to both fragments of PTHrP (Table 2).

Through histomorphometry carried out in vertebrae L1-L5 we observed that the diabetic mice showed a reduction in total trabecular volume (BV/TV), in average thickness (Tb.Th) and in the number of trabeculae (Tb.N), and an increase in trabecular separation (Tb.S), parameters which were normalised after the treatment with the PTHrP peptides (Table 3). The von Kossa stain allows the clear visualisation of these alterations in trabecular bone in the vertebrae in each of the experimental groups studied (Figure 2).

In the femur of the diabetic mice, we analysed the gene expression involved in the activation of the Wnt/β-catenin pathway. We observed that the levels of mRNA of the Wnt3a ligand, of the Fz2 receptor and of the co-receptors of Lrp5 and Lrp6, as well as those of Ccnd1 (a final target gene of this pathway) were reduced in these mice (Figure 3A). Furthermore, in the osteoprogenitors of the in the bone marrow (BMMCs) of the long bones we found a lower protein expression of β-catenin (Figure 3B). These deleterious effects of diabetic status on effectors of the Wnt/ β-catenin pathway were counteracted by the administration of PTHrP *in vivo* (above all by the N-terminal fragment) and *in vitro* (Figures 3A and 3B).

Given that DM is associated with an increase in oxidative stress, we analysed the production of oxidised proteins in the femurs of the diabetic mice[35]. These animals had an increase in oxidised proteins with respect to the controls, which showed a tendency to normalisation after treatment with PTHrP (1-36), but not with PTHrP (107-139) (Figure 3C).

Alterations in bone mass and structure associated with a deficit of IGF-I in mice and its modulation by PTHrP

The Igh1-null mice showed a significant reduction in BMD and BMC with respect to the control mice in the total body, femur and spine (L1-L5) (Figure 4A). At the end of the period of study (day 14) the Igf1-null mice showed a lower gain in bone mass in the total body, but greater in the femur and the spine which respect to the controls (Figure 4B). The treatment with both PTHrP peptides produced a significant increase in bone mass in the total body and in the femur of the Igf1-null mice (Figure 4B). Through a histomorphometric analysis, a general change was observed in the structural parameters evaluated in the L1-L5 vertebrae of the Igf1-null mice compared to the controls. Treatment with the PTHrP peptides normalised the BV/TV and the Tb.Th in these animals (Table 4).

In the Igf1-null mice we found in the femur a reduction in an initial gene and another final gene, key to the activity of the canonical Wnt, Wnt3a and Cx43 pathway, which was partially compensated for by treatment with the PTHrP peptides (Figure 5A).

In addition, we wanted to confirm whether PTHrP might exert osteogenic actions autonomously at the cellular level in the absence of IGF-1. In order to do this we used BMMC cultures from control and Igf1-null mice treated *in vitro* with both PTHrP peptides. The cultures from Igf1-null mice showed a lower capacity for mineralisation compared with the controls, which was not affected by the treatment with either PTHrP peptides (Figure 5B).

Discussion

Osteogenic effects of PTHrP in murine model of DM induced by STZ

In this study we observed a loss of weight in diabetic mice, possibly due to lipolytic action and loss of muscle induced by the drug STZ[36,37]. Using DXA we corroborated this finding with the decrease observed in the percentage of periosteal fat in the total body and the long bones of the diabetic mice. In these locations we observed, furthermore, a reduction in bone mass at 4 weeks from the instigation of DM. The treatment with PTHrP peptides compensated for this osteopenia, in accordance with earlier observations in this model of DM1 after the administration of analogues of PTH and PTHrP[25,26,38,39].

The histomorphometric analysis of the L1-L5 vertebrae showed a reduction in BV/TV and other trabecular parameters (Tb.Th, Tb.N and Tb.S) in diabetic mice, in accordance with observations in

Table 2. Values of bone mass and periosteal fat in the long bones, spine and total body of control an diabetic mice, with and without treatment PTHrP (1-36) or PTHrP (107-139)

		Control	Diabetic (DM)	DM+PTHrP (1-36)	DM+PTHrP (107-139)
Femur	BMD	0.123±0.001	0.103±0.0009**	0.106±0.001	0.119±0.001##
	BMC	0.046±0.001	0.041±0.0005**	0.042±0.0007	0.047±0.001#
	%Fat	19.45±2.376	11.56±0.409**	17.76±0.248##	13.34±0.441#
Tibia	BMD	0.084±0.002	0.076±0.0004**	0.086±0.002#	0.086±0.002##
	BMC	0.046±0.001	0.042±0.0007*	0.045±0.001#	0.046±0.0004##
	%Fat	18.94±0.909	14.98±0.485**	16.78±0.171##	18.64±0.930##
Column	BMD	0.077±0.003	0.074±0.001	0.074±0.0009	0.076±0.0007
	BMC	0.092±0.006	0.057±0.011*	0.089±0.001#	0.094±0.003#
	%Fat	18.78±1.084	11.06±0.175	10.4±0.175#	11.21±0.606
Total body	BMD	0.064±0.001	0.056±0.001**	0.067±0.001##	0.067±0.002##
	BMC	0.901±0.029	0.864±0.017	0.892±0.019	0.892±0.001
	%Fat	18.78±1.084	12.82±0.582**	13.56±0.597	12.92±2.143

BMD (g/cm^2); BMC (g). The values are the mean ± SEM of 5 mice/groups. *p<0.05; **p<0.01 vs control; #p<0.05; ##p<0.01 vs DM.

the other model of DM1 induced by STZ in mice[40]. On the other hand, recent data from a histomorphometric analysis of biopsies from the iliac crest of patients with DM1 did not indicate significant alterations in the trabecular structure compared with a healthy control group, although there is a coherent trend with results obtained in the vertebrae of diabetic mice in our study[41]. However, it is interesting to note that in these diabetic patients the samples were obtained before the appearance of complications associated with DM. Our results demonstrate the capacity of the PTHrP peptides to attenuate alterations in the vertebral trabecular structures produced by DM in mice, confirming previous findings[25,26,44].

Recent data from our group have shown changes in the Wnt/β-catenin pathways in the bone of mice with DM1 induced by STZ, associated with a reduction in sclerostin corresponding to a higher rate of osteocyte apoptosis in the tibia of these mice[42]. On the other hand, an overexpression of Sost and Dkk1 (inhibitors of the Wnt canonical pathway) was found in the tibias of diabetic mice[43]. In humans, high levels of sclerostin and a reduction in β-catenin have been found in patients with DM2[44]. The results of this work show an alteration in the expression of the canonical genes for the initial stages of the Wnt pathway in the bone of diabetic mice, in contrast with that observed in diabetic rats[43]. So, the alterations in the components of the Wnt pathway in a diabetic state appear complex and species-dependent.

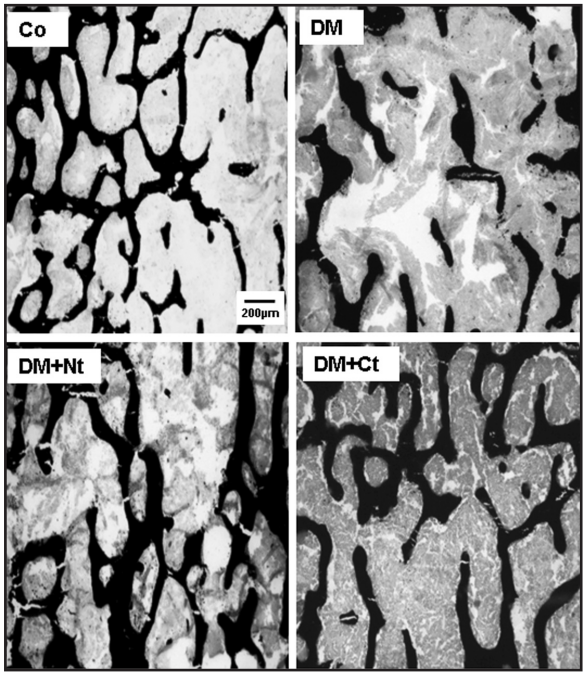
The hyperglycemic state associated with DM1 causes an increase in the reactive species of oxygen (ROS), which produces an increase in protein carbonylation[35,45]. The increase observed in carbonylated proteins in the femur of diabetic mice is reduced in those treated with the N-terminal fragment of PTHrP. Similarly, the ability of PTH to reduce the production of ROS in BMMCs in the femur of old mice has been described[46]. An excess of ROS in diabetic bone affects osteoblastogenesis -causing the differentiation of the BMMCs towards adipogenesis-[47,48] and the osteoblast function, diminishing the expression of Runx2, AP and Col1α-[49], while also activating the transcription of FoxO which antagonises the canonical Wnt signalling[50]. Thus, we found a reduction in β-catenin in cultures of BMMCs originating from the long bones of diabetic mice. In this respect, in a model of non-obese diabetic mouse (similar to the model of DM1 by STZ) it was observed that there was a suppression of the PI3K/AKT pathway in osteoprogenitors cells which could contribute to the destabilisation of the β-catenin in these cells[51]. In humans, a mutation of the Sirt1 gene, directly related to the development of DM1[52], has been described, which is of interest since the SIRT1 protein promotes the translocation to the nucleus of β-catenin in osteoprogenitors cells[53].

Table 3. Alterations in histomorphometric parameters in trabecular bone (vertebrae L1-L5) of diabetic mice treated, or not, with PTHrP

Parameter	Control	Diabetic (DM)	DM + PTHrP (1-36)	DM + PTHrP (107-139)
BV/TV (%)	36.93±1.64	22.21±1.6**	37.19±2.76##	37.43±3.7##
Tb.Th. (µm)	85.49±4.53	59.91±2.24**	83.93±4.81##	88.24±2.91##
Tb. N. (mm^{-1})	2.26±0.09	1.77±0.09*	2.32±0.07##	2.30±0.15#
Tb. S. (µm)	146.93±9.71	225.97±12.07**	130.12±1.45##	130.52±12.49#

BV/TV: total trabecular volume; Tb.Th.: average trabecular thickness; Tb.N.: number of trabeculae; Tb.S.: trabecular separation. The values correspond to the mean ± SEM of 5 mice/group *p<0.05, **p<0.01 vs control; #p<0.05, ##p<0.01 vs DM.

Figure 2. Alterations in trabecular bone in the vertebrae (L1-L5) of diabetic mice with or without treatment with peptides of PTHrP. Shown are representative images obtained by optical microscope (4x) of histological sections of vertebrae of control (Co) or diabetic (DM) mice, treated, or not, with Nt (Nt) or C-terminal (Ct) PTHrP, after being set in methacrylate and with von Kossa stain, showing trabecular structure



Our findings demonstrate that PTHrP (predominantly its N-terminal fragment) is capable of counteracting, at least partly, the oxidative stress and alterations in different active components of the Wnt pathway as part of its osteogenic actions in diabetic bone.

Osteogenic effects of PTHrP (1-36) and osteostatin in a mouse model deficient in IGF-I

The IGF system plays a determining role in the regulation of somatic growth. It has been suggested that a reduction in the production and/or activity of IGF-I may contribute to the loss of bone mass associated with age[54]. However, it has also been speculated that this reduction would cause a lower level of bone remodelling and thus preserve the solidity of the long bones in this situation[55]. IGF-I increases the periosteal bone formation, but its effects in trabecular bone are variable[16,56,57]. The differences observed in the skeletons of mice deficient in IGF-I could be the consequence of the dual effect of this factor on osteoblastogenesis and osteoclastogenesis and its relative impact according to bone location[16].

In this work we used a mouse model deficient in the expression of Igf1 which shows significant alterations in the mass and structure of the trabecular bone in the vertebrae, compensated for in part by both PTHrP peptides. It is worth mentioning the anabolic effects of PTH observed in the trabecular bone of mice deficient in IGF-I synthesised in the liver[58]. The low resorptive activity associated with IGF-I deficiency could facilitate the manifestation of an anabolic action of PTHrP in trabecular bone[16,59]. In fact, anabolic effects of both N- and C-terminal PTHrP fragments have been described in trabecular bone in the femur of mice diabetic due to STZ, with low levels of bone remodelling[25,26].

We observed significant changes in various components of the canonical Wnt pathway compatible with alterations in bone remodelling in mice deficient in IGF-I. Previous data in mice with a deficit of IGF-I in osteocytes showed a marked deficiency in bone development and in the response to mechanical stimulation, associated with a deficient activation of the Wnt pathway[60,61]. In our study we found that the administration of PTHrP (1-36) or osteostatin partly corrects the alterations observed in the canonical Wnt pathway in mice deficient in IGF-I. Similarly, as our data show, both PTHrP (1-36) and the native C-terminal fragment of PTHrP (107-139) act on this metabolic pathway in mice diabetic due to STZ[25,26,42].

In addition, we found that the BMMCs of mice with IGF-I deficit showed lower osteogenic capa-

Table 4. Alterations in histomorphometric parameters in the L1-L5 vertebrae of Igf1-null mice treated, or not, with PTHrP

Parameter	Control	Igf1-null	Igf1-null+Nt	Igf1-null+Ost
BV/TV (%)	26.1±1.5	16.5±0.9**	22.6±3.3#	22.85±0.2#
Tb. Th (μm)	54.5±2	45.9±1.8**	65.4±3**,##	52.5±2.5#
Tb. Sp (μm)	156±6.8	214.7±19.3**	230.6±27.9**	213.2±9.24**
Tb. N (1/mm)	4.7±0.1	3.7±0.2**	3.4±0.3**	3.6±0.23**

BV/TV: total trabecular volume/total volume; Tb.Th: trabecular thickness; Tb.N: number of trabeculae; Tb.Sp: trabecular separation. Nt, PTHrP (1-36); Ost, osteostatin. The values are the mean ± SEM of 6 mice/group. **p<0.01 vs control; #p<0.05; ##p<0.01 vs Igf1-null.

city than the control mice. A similar result was obtained in mice with a deficit of Igf1r in mature osteoblasts[62,63]. Furthermore, these BMMCs showed a lack of response to PTHrP *in vitro*, indicating that IGF-I is essential for the action of PTHrP on these osteoprogenitor cells.

These findings, overall, show that PTHrP, predominantly through its N-terminal domain, is capable of modulating the canonical Wnt pathway in relation to its osteogenic actions in a diabetic situation. Furthermore, a functional IGF-I system is necessary for at least a part of the osteogenic actions of PTHrP (1-36) and osteostatin in the mouse skeleton.

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Figure 3. Effect of PTHrP on the Wnt/β-catenin pathway in the long bones of diabetic mice. (A) Changes in the expression of genes related to the Wnt canonical pathway (analysed by real time PCR) in the femurs of control (Co) and diabetic (DM) mice, treated or not with the N-terminal (Nt) or C-terminal (Ct) fragments of PTHrP. (B) Representative autoradiography of the changes in the expression of β-catenin in BMMCs extracted from the femurs and tibias of these mice, cultivated for 14 days in an estrogenic medium, in the presence or absence of each of the PTHrP peptides (100 nM). The average relative intensities of the β-catenin signal, normalised to that of β-actin for each of the experimental condition, to the control in a representative experiment, are shown. (C) The effect of PTHrP on the oxidation of proteins in diabetic mice. Measure of the carbonylated proteins in the femur of control and diabetic mice, treated or not with the PTHrP peptides. The results in A and C correspond to the mean ± SEM of the values obtained in 5 mice in each experimental condition. *p<0.05, **p<0.01 vs Co; #p<0.05, ##p<0.01 vs DM

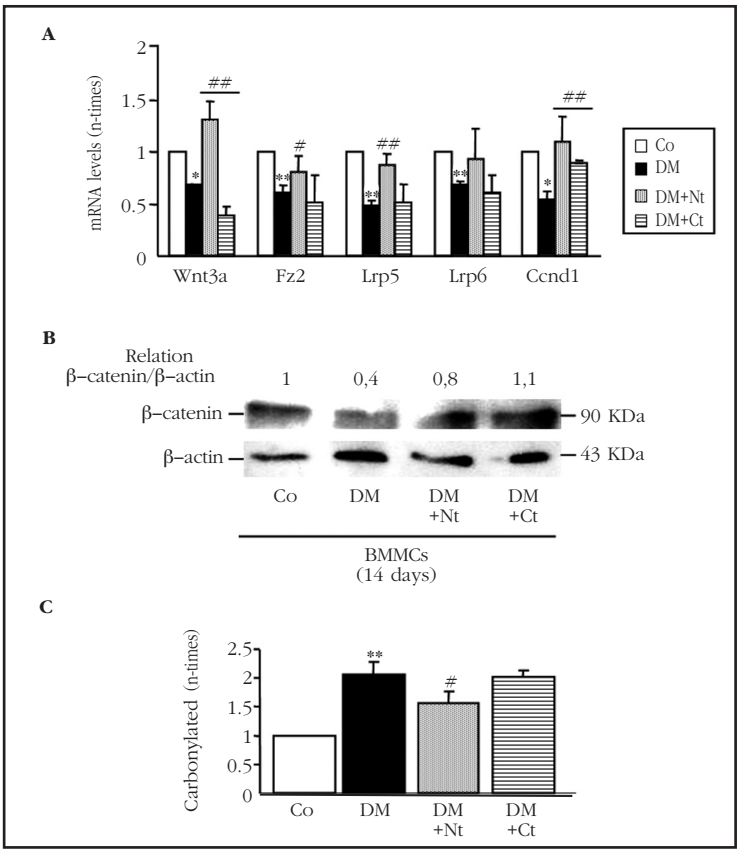
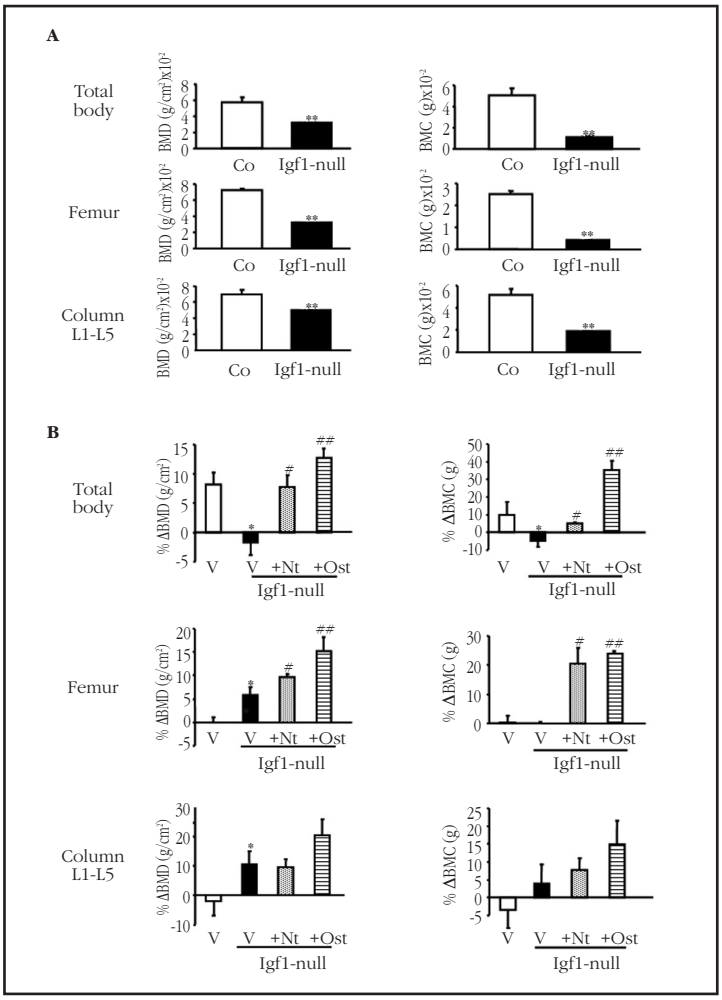


Figure 4. Description of the changes in bone mass of Igf1-null mice treated or not with PTHrP. (A) Values of BMD and BMC of control (Co) and Igf1-null mice at the start of the study (day 0) in total body, femur and spine. (B) Increases (**Δ**) in % of the values of BMD and BMC since the start (day 0) until the end of the study (day 14) for each of the genotypes, and effect of treatment with N-terminal PTHrP (Nt) or with osteostatin (Ost) (or vehicle, V). The values correspond to the means ± SEM of 6 mice for each experimental condition. **p<0.01 vs Co (A); *p<0.05 vs V-control; #p<0.05; ##p<0.01 vs V-Igf1-null (B)



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

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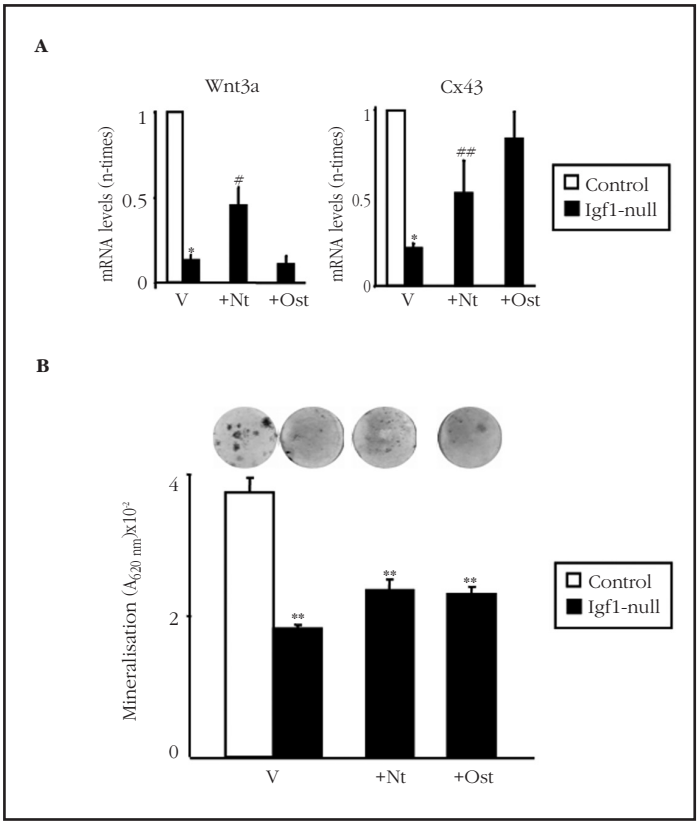
Figure 5. (A) Changes in factors related to the canonical Wnt pathway in Igf1-null mice, treated or not with PTHrP. Gene expressions (evaluated by real time PCR) of Wnt3a and Cx43 in the femurs of these mice, and the effect of treatment with PTHrP (1-36) (Nt) or osteostatin (Ost) (or vehicle, V). The values represent the means ± SEM of 6 mice/group. *p<0.05 vs V; #p<0.05; ##p<0.01 vs V-Igf1-null. (B) Alteration in the mineralising capacity of BMMCs in Igf1-null mice. The BMMCs of 2 Co mice or 5 gf1-null mice were cultivated for 16 days, with or without (saline vehicle, V) PTHrP (1-36) (Nt) or osteostatin (Ost) (100 nM). The mineralisation was evaluated by staining with alizarin red S (representative images are shown). The values represent the means ± SEM for 7 culture wells per experimental condition. **p<0.01 vs V-control



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Endocrine regulation of energy metabolism by bone

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Summary
The classical functions of bone are the maintenance of phosphorus-calcium homeostasis, damage repair, as well its structural function which allows locomotion and protects the vital organs. The recent discovery of new functions for bone in the regulation of energy metabolism suggest that bone may be an endocrine organ.
In the last decade, different genetic and molecular studies carried out in mice have determined that osteocalcin increases the secretion of insulin, and sensitivity to it, by increasing the secretion of adiponectin, stimulates the proliferation and the better functioning of the beta cells, promotes the reduction of fatty mass and an increase in the consumption of energy.
These findings demonstrate the existence of a reciprocal regulation between bone and energy metabolism, mediated by osteocalcin. The recognition of the metabolic role of osteocalcin is a significant discovery in the field of osteology and endocrinology, bringing the possibility of new therapies in the treatment and prevention of metabolic diseases such as diabetes mellitus, sarcopenia, obesity and osteoporosis.

Key words: *osteocalcin, bone, endocrine organ, energy metabolism, insulin, diabetes mellitus, obesity.*

Introduction

The classical functions of bone are the maintenance of phosphorus calcium homeostasis, the repair of damage to the bone, as well as a structural function which allows locomotion and protects vital organs[1]. The bone is a dynamic tissue in constant change through bone remodelling, and which requires a great quantity of energy to perform this process[1-3].

Osteoporosis is the most frequent disease caused by changes in bone remodelling and is related to an increase in bone resorption or a decrease in its formation. The clinical observation that osteoporosis happens as a consequence of gonad failure and that being overweight protects against the development of osteoporosis suggests a hypothesis that appetite or body mass, reproduction and bone mass may have a hormonal regulation mechanism in common. This conjecture, and the recent discoveries of the new functions of bone in energy metabolism, suggest that bone may be an endocrine organ.

In the last few decades, numerous clinical trials have demonstrated that leptin, a hormone deriving from adipocytes, regulates the appetite and exerts a bimodal antagonistic effect on bone remodelling. To achieve this, two different hypothalamic pathways are used, the sympathetic nervous system (SNS) and the cocaine and amphetamine regulated transcript system (CART), which act directly on the osteoblasts[2].

Lee et al., in different genetic and molecular studies carried out in mice, determined that osteocalcin increases the secretion of insulin and its sensitivity by raising the secretion of adiponectin; and that it also stimulates the proliferation and functioning of the beta cells, at the same time as promoting the reduction of fat mass and an increase in the consumption of energy[4,5]. These findings demonstrate the existence of a regulatory relationship between bone and energy metabolism, mediated by osteocalcin (OC).

In some previous studies carried out in humans, different markers for low bone mass were detected in diabetic patients, among which was OC, but until very recently, research had not been carried out in these patients to determine its metabolic functions. Recently, an association has been found in adults between concentrations of OC and markers for metabolic syndrome and obesity, confirming the existence of an inverse relationship between OC and fat mass and levels of glucose[6].

This reciprocal relationship between bone and energy metabolism, with the recent recognition of the metabolic role of OC, is a significant discovery in the field of osteology and endocrinology, making possible new therapies for the prevention and treatment of metabolic diseases such as diabetes mellitus, sarcopenia, obesity and osteoporosis.

Bone is an endocrine organ

An endocrine organ may be defined as one capable of regulating development, integrating the functions of diverse organs and tissues, and coordinating the metabolic process of an organism by means of the synthesis and release of hormones secreted into the circulation. These regulatory functions are performed through feedback mechanisms in which the concentrations of the hormone itself indicate the necessity of increasing or reducing its production. This function is a fundamental characteristic of endocrine organs.

Bone remodelling is a biphasic process which occurs sequentially and in a balanced way. It consists of destruction or resorption followed by formation of new bone matrix[7,8]. This process allows the constant maintenance of bone mass throughout adulthood, and is essential for the maintenance of bone architecture to meet mechanical requirements, the repair of damage which occurs in daily exercise and the maintenance of the homeostasis of the phosphorous-calcium metabolism, such that remodelling constitutes a true homeostatic function[7-9]. A great many homeostatic functions, such as appetite and reproduction, are controlled by the hypothalamus. It is not strange to imagine that remodelling could, at least in part, be controlled by a central mechanism[9].

Bone remodelling requires a large and continuous supply of energy to the bone cells[1]. To cover this huge energy cost there needs to be coregulation between energy metabolism and bone. In other words, bone remodelling may play a significant role in how glucose and energy are managed in the body[7].

These two biological aspects (the establishment of bone remodelling as a homeostatic function and its central control), along with their participation in the regulation of energy and glucose, suggest the hypothesis that there is regulatory coordination between bone remodelling and energy metabolism, probably through a central mechanism[8,9].

In the identification of hormones which may regulate the formation of bone, we start with two essential clinical facts. First, osteoporosis originates with gonad failure[10], and second, being overweight appears to protect against osteoporosis[11-13]. These two observations suggest the existence of a common regulatory mechanism between appetite, reproduction and bone mass. In attempting to determine the regulatory hormone or hormones, it has been observed that leptin is the only one which significantly influences all three functions.

It has been established that there are different phases in the central regulation of bone remodelling. The process starts with the emission of afferent signals from the adipocytes to the hypothalamus, in which leptin has an important role. It continues with a complex hypothalamic neural phase in which numerous neuropeptides participate, and from which originate efferent signals towards the adrenergic β2 receptors in the osteoblasts. As a consequence there are changes in transcription factors and clock genes which affect osteoblastogenesis. The last phase is the regulation by bone of the adipocytes, probably through the release of OC. The adipocytes may, in turn, regulate the proliferation and differentiation of the osteoblasts[14] (Figure 1).

Osteocalcin (OC)

Bone and energy metabolism appear to have a reguation which is linked by a central component, which is fat. To validate this hypothesis, a mediator is necessary which, originating in bone, is able to regulate energy metabolism. This mediator is OC.

The strategy for demonstrating this theory requires the identification of genes specific to the osteoblasts, to produce mice with deletions and to study the metabolic phenotypes[15]. Lee et al., in different experiments *in vitro*, confirmed that osteoblasts secrete a substance which affects the pancreatic cells and the adipocytes, and which appears to regulate glucose metabolism[4]. The co-cultivation of osteoblasts of wild-type mice with pancreatic islets increased by up to 500% the expression of the gene for insulin and of the markers for cell cycle progression in the islets. Karsenty et al., were the first to propose, in 2006, that there was endocrine regulation of energy metabolism in the skeleton[1].

The observation that a mouse deficient in protein specific to the osteoblast cell line, OC, (OC-/-), showed an abnormal quantity of visceral fat, led to the hypothesis that this was the hormone secreted by the osteoblasts which affected glucose metabolism[4].

OC is one of the few proteins specific to the osteoblasts which has numerous characteristics of a hormone. It is a molecule of low molecular weight (5,700 Da) produced by the osteoblasts[4]. It is present in all vertebrates, and is considered to be a marker for differentiation to mature osteoblasts. It is secreted into the circulation and, since its identification 30 years ago, has been considered to be the main constituent of the extracellular matrix, where it bonds with hydroxyapatite by means of three gamma-carboxylate residues, called Gla residues. This carboxylation offers an opportunity for regulation[16]. Surprisingly, although it is the most abundant non-collagen protein (15% in bone), it is not involved in bone formation[17].

Mice without OC have high levels of glucose, low levels of insulin and a reduction in insulin secretion stimulated by glucose, in comparison with those with the wild genotype[4,20,21] (Figure 2). In the pancreas, the size of the islets, the mass of the beta cells and the amount of insulin were reduced. In addition, fat mass, the number of adipocytes and levels of triglyceride were increased. The expression of adiponectin and its molecular targets were reduced, and a subcutaneous infusion of recombinant OC in wild mice would produce an increase in insulin and in its sensitivity, and improve the expression of the insulin genes[4].

Subsequently, mice were obtained which had an absence of genes which are expressed preferentially in the osteoblasts. The first gene was Esp, which codes for the receptor of the tyrosine phosphatase protein (OST-PTP) present in mother cells, Sertoli cells and in osteoblast, and which are not expressed in the beta cells of the pancreas or in adipose tissue[2,4,18]. Those mice without Esp (Esp-/-) had larger and more numerous pancreatic cells than wild mice, proliferating by 60% to 300% in mice from the age of 5 days to one month. The increase in levels of insulin and insulin sensitivity was due to the capture of glucose in the muscle, brown fat, white fat and liver, and to the increase in adiponectin (the only adipokine affected, which was doubled in these mice), as well as an increase in the expression of other target genes of adiponectin, which increased threefold, such as the acyl-CoA oxydase gene, the gene for the receptor activator of the proliferation of alpha peroxisome (PPAR alpha), and the gene for the uncoupling protein 2 (UCP 2). On the other hand, those genes which coded for insulin-sensitising enzymes, as well as for adiponectin and insulin, were reduced in those mice without OC[19]. In mice without Esp an up to threefold increase was observed in the mitochondrial area and in proteins associated with biogenesis. In spite of insulin being a lipogenic hormone these mice were thinner due to an increase in energy use, evidenced by an increase in the expression of PGC1-alpha and of the uncoupling protein 1 (UCP 1), without the appetite being affected. The reduction in fat mass was restricted to visceral fat, and triglyceride levels were lower.

In order to confirm that the deletion of Esp would protect against the development of diabetic symptoms studies were carried out in mice in which hyperphagy was induced through hypothalamic structural lesions, destruction of beta pancreatic cells or feeding with a fatty diet. In the mice without Esp, it was confirmed that there was a lower weight gain and that no glucose intolerance or insulin resistance were developed[20].

The fact that the mice without OC have a phenotype contrary to those without Esp suggests that the two genes are in the same pathway, although they do not physically interact (Table 1). Lee reported that 90% of the OC was bonded with hydroxyapatite, while in the mice without Esp this was only 74%. This fact raises the possibility that OST-PTP, regulated by the Esp gene, would be in some way involved in the gamma-carboxylation of OC[21].

OC is secreted by the osteoblasts following many posttranslational modifications. These modifications include the excision of a pre-propeptide and gamma-carboxylation, dependent on vitamin K, of the three glutamic residues in the Gla residues[22]. This gamma-carboxylation is essential in order for the protein to have a high mineral affinity and to enable OC to attract calcium ions and incorporate them into the hydroxyapatite crystals, which form 70% of bone[22].

There is a high proportion of non-carboxylated OC in circulation, while a greater proportion of carboxylated OC is found in the bone matrix. However, not all the residues of the three glutamic acids are completely carboxylated and incorporated into the crystals, and the degree of carboxylation varies[23]. Vitamin K is a cofactor for the enzyme glutamate carboxylase which is required for the carboxylation of the proteins which contain Gla in the coagulation cascade and for the carboxylation of OC. Carboxylation or its absence

Figure 1. Scheme for central regulation of bone remodelling. Adapted from Rosen[14]

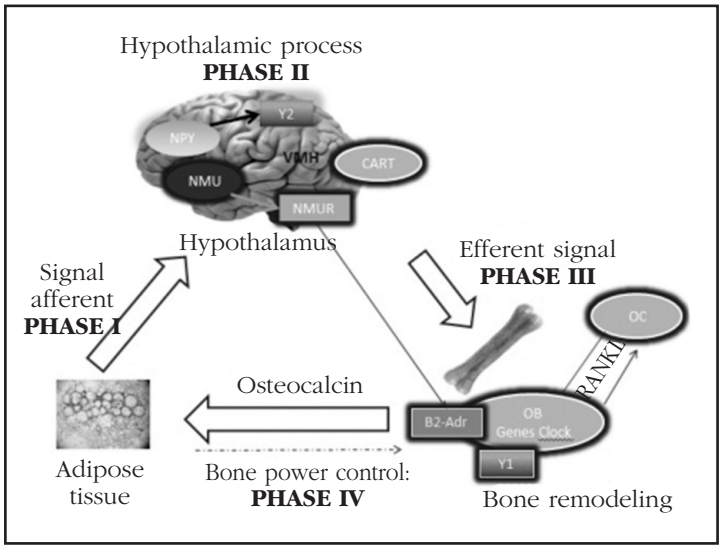
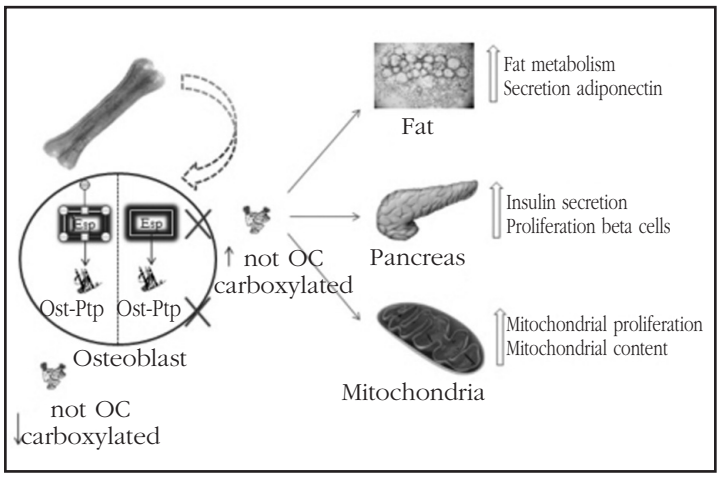


Figure 2. Integrative physiology: new metabolic pathway of osteocalcin. Adapted from Kim et al.[19]



makes OC susceptible to release by the osteoblasts into the circulation. Experiments in vitro carried out in isolated islets and in primary adipocytes, have revealed that the carboxylated form is inactive while the non-carboxylated form is the active form[4,24].

As we have already commented, the (Esp-/-) mice and mice treated with non-carboxylated OC resulted in a protector phenotype, as opposed to the (OC-/-) model. Alternatively, the overexpression of OST-PTP resulted in a phenotype identical to the (OC-/-) mice[4,25].

The metabolic changes in mice with a deletion of the insulin receptor in the osteoblasts provides evidence of a link between the action of the insulin in bone remodelling and glucose homeostasis.

The signalling of the insulin in the osteoblasts inhibits carboxylation, which means that it is a positive regulator of metabolic activity. OST-PTP inhibits insulin signalling, such that the metabolic phenotype of mice without Esp is secondary to an increase in insulin signalling in the osteoblasts and to the secondary increase in non-carboxylated OC[25].

Insulin promotes the ability of osteoblasts to improve bone resorption. The expression of OPG is reduced, through Fox01, and this promotes bone resorption and, in particular, the expression of Tcigr 1 which codes for a proton pump subunit which contributes to the acidification of the extracellular bone matrix[21]. The acidic pH of around 4.5 generated during bone resorption is sufficient to de-carboxylate and activate the molecules of OC (Gla-OCN) stored in the extracellular bone matrix. Sufficient quantities of non-carboxylated OC are produced to induce the expression of the beta cells and to stimulate in them the expression of insulin, just as with treatment with recombinant OC[25]. This shows a pH-dependent activation mechanism for this hormone, and identifies insulin signalling as a critical link between bone remodelling and energy metabolism[25,26].

The data obtained in numerous studies in humans regarding the relationship of OC with glucose homeostasis and, on the one hand, energy metabolism and, on the other, bone metabolism, are highly significant. In the first area, the findings are very interesting. The mice models show OC to be a hormone which positively regulates the production of insulin and its sensitivity. Low levels of OC are associated with diabetes mellitus, glucose intolerance and insulin resistance. Additional studies have established that low levels of OC reduce glycosylated haemoglobin (HbA1c), insulin when fasting and insulin resistance by means of the homeostasis model assessment (HOMA), independently of the presence of diabetes mellitus[6,27-29]. These data suggest that an increase in the levels of OC could be a therapy for the treatment of diabetes in the future.

It has also been found that levels of OC in circulation are inversely correlated with parameters of adiposity, such as body mass index (BMI) or fat mass, and with levels of blood glucose in men and women of different ethnicities[6,20,30].

Levels of circulating OC have also been associated with a number of lipid abnormalities, and are positively correlated with levels of adiponectin in postmenopausal women[20]. More generally, obese people have less OC than those who are not obese, and those with diabetes mellitus type 2 have less OC than non-diabetics[20,30].

The dynamic production of OC as a marker for energy and glucose metabolism has begun to be tested. For example, it has been seen that a significant loss of weight causes a decrease in the

Table 1. Characteristics of (Esp-/-) and (OC-/-) mice. Adapted from Wah[21]

Esp-/-	OC-/-
• Neonatal death due to hypoglycaemia and hyperinsulinemia • Increase in insulin sensitivity • Increase in insulin secretion • Increase in beta cells and pancreatic area • Increase in adiponectin • Increase in energy use • Reduction in body weight and accumulation of fat • Low levels of triglycerides and fee fatty acids • Significant increase in non-carboxylated OC	• Hyperglycemia • Decrease in insulin sensitivity • Decrease in secretion of insulin • Decrease in beta cells and pancreatic area • Decrease in adiponectin • Decrease in energy use • Abnormally fat • Increase in triglycerides • OC not expressed

homeostasis model assessment for insulin resistance (HOMA-IR), as well as an increase in levels of OC in obese children[31]. In non-diabetics, loss of weight through diet and exercise increases levels of OC in parallel with the decrease in visceral fat, and increases sensitivity to insulin[32,33]. Lower levels of OC have also been correlated with acute myocardial infarction.

In terms of the second area, it has been widely reported that the alterations in glucose metabolism may be a risk factor in loss of bone mass and fractures in humans[34]. In a transverse study it was found that osteoporotic women, after 6 months or more of treatment with alendronate had lower levels of non-carboxylated OC than those who had not been treated. There are scarcely any studies which establish the effects of bisphosphonates on the homeostasis of glucose or insulin sensitivity[35]. Treatment with alendronate or raloxifene (selective estrogen-receptor modulator) for 12 weeks reduced the biochemical markers for bone remodelling, including OC[36].

It would be an important step to confirm whether the antiresorptive drugs, which are the main treatment for osteoporosis, may in some cases have a negative effect on glucose tolerance, as well as the effect of the anticoagulants which affect vitamin K[25].

Lastly, it is also worth highlighting the possible relationship which appears to exist between OC and the endocrine regulation of male reproduction through the regulation of androgen production[37,38].

Conclusions

Recent models in mice suggest a new role for bone in which it is the source of a hormone, non-carboxylated OC, which affects energy metabolism, insulin resistance, obesity and the development of diabetes. So, an organ with an endocrine function, such as fat, is mediated by the endocrine function of another organ, the skeleton. Studies which should be carried out, based on this physiological integration, should review other new areas which have not yet been studied.

There are increasing numbers of studies which are establishing that many aspects of the biology of OC are similar in mice and humans, and which are attempting to determine whether or not the endocrine system of mice is different to that in humans.

The possible role of adiponectin, leptin, OC and insulin, which appear to integrate the functions of the adipose, bone and pancreatic tissues to regulate the metabolism of glucose, bone remodelling, energy and lipid metabolism, remains to be clarified.

We may consider that the skeleton, as an endocrine organ, is involved in the overall co-ordination of energy use, through interaction with other tissues. The identification of OC, and its regulation and different effects, has provided the basis for the development of potential therapeutic applications for metabolic diseases such as diabetes, sarcopenia or obesity.

Both authors declare that there are no conflicts of interest.

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Clinical case discussion: therapeutic holiday, yes or not?

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Presentation

We introduce a new, special type of document, in which, within the Journal itself, we will debate a currently controversial theme which will allow the reader to reflect and, above all, participate by contributing their opinions in the form of letters to the Editor.

On this occasion, we address the matter of treatment holidays, using a clinical case. Two reasoned opinions, in favour and against, are expounded below, with the sole aim of setting out the arguments and stimulating the contribution of readers to the debate.

Clinical case

A female patient currently 63 years of age, who attended a clinic for the first time in July 2004 at the age of 53. She was referred by her family doctor due to a clinical picture of back pain, which had developed over a period of several months. At this first visit a lateral dorsal-lumbar spine X-ray was requested (Figure 1) which showed the existence of a lumbar vertebral fracture.

In terms of personal history, the patient had an early menopause at 35 years of age, without there being any disease to which would have caused it, for which hormone replacement therapy for 5 years was indicated. Similarly, hypercholesterolemia was diagnosed, for which the patient followed a diet and took simvastatin irregularly. The patient does not consume tobacco or alcohol, is sedentary and has no other history of interest. She does not take any other medication, apart from the aforementioned statin.

The results of the tests carried at the first visit were completely normal. The data relating to bone mineral metabolism are shown in Table 1. The levels of vitamin D, measured as 25-hydroxycolecalciferol (25-HCC), were 22 ng/mL. The evaluation of risk at 10 years using both the FRAX® scale and Qfracture® is shown in Table 2.

The densitometry values are shown in Table 3 and their development over time in Figures 2 and 3.

Treatment indicated for the patient was in the form of general measures such as walking daily for an hour, increasing intake of skimmed milk products, calcium and vitamin D supplements, and alendronate with vitamin D weekly. The patient was evaluated by the rehabilitation service which provided physiotherapy for a period of 2 months, reducing her back pain. The patient has continued with the treatment indicated for 10 years, which she tolerates well, without secondary effects, that she occasionally forgets the calcium and vitamin D supplements. She has had no falls or fractures during this period of treatment.

At the last visit, which occurred in February 2014, after 10 years of treatment, she brought along a report from her family doctor which asked our opinion regarding the possibility of discontinuing the patient's treatment and giving her treatment holidays.

Reasoned arguments

A) Reasons for maintaining this patient's treatment (NO to treatment holidays)

Manuel Sosa Henríquez and Mª Jesús Gómez de Tejada Romero

Our main aim in instigating treatment for osteoporosis is to avoid the appearance of fragility fractures[1], which is the clinical complication of this disease, whether it the first fracture before it has even been presented or, if it were already there, successive ones.

Equally important are the consequences of the fractures, that is to say the appearance of pain and the worsening of the quality of life. So, with the treatment, we are also attempting to avoid or reduce this pain or improve the patient's quality of life[2].

The purely clinical reasons (which is to put economic reasons and the patient's personal reasons to one side) for discontinuing a particular treatment, for osteoporosis or for any other disease, in our view are:

1. Due to the disease being cured. Hence, we stop an antibiotic when the infection ceases, or an anti-inflammatory once the inflammatory process is cured. This is not the case with osteoporosis, which is a chronic disease, with profound microstructural changes which are not cured.

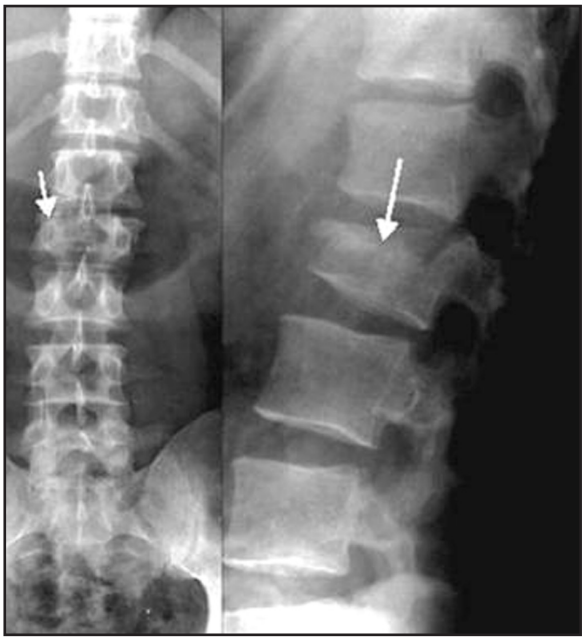
2. Due to the loss of effectiveness of the drug used. Using the same example as before, some micro-organisms may develop resistance to an antibiotic, which, having initially been useful, stops being so. In the field of osteoporosis this is much more complicated to define, given that, on the one hand, fractures have a multifactorial etiopathology and on the other, the drugs we have available reduce the risk of fracture but do not eliminate them completely. To simplify the response, we are assuming "treatment failure" criteria as indicated by Díez Pérez et al[3].

3. In some cases, a limit is established before the use of a drug. Hence, teriparatide may be used over a maximum period of 2 years, according to the indication given in its data sheet. Or zoledronate, in which, according to recently published data, after 5 years of use the reduction in the risk of fracture is the same whether it continues to be administered or is discontinued[4].

4. Another reason for discontinuing a drug is the appearance of secondary effects which overcome the beneficial effects of its use against the disease for which it is prescribed. For example, in the treatment of osteoporosis with SERMs the appearance of thromboembolic phenomena necessitates its cessation and substitution by another type of antiosteoporotic drug.

Taking these reasons into account, in the case which occupies us is there any reason for stopping treatment in this patient? The treatment has been maintained over 10 years, and even with her being a high risk patient, with a previous vertebral fracture, in all this time she has not suffered any new fractures. Therefore the treatment up to this point in time has been successful, having achieved exactly what was expected of it. In addition, the patient is asymptomatic, her biochemical markers for bone remodelling are normal and her bone mineral density has not stopped increasing since the therapy was initiated.

Figure 1. Lateral and anteroposterior X-rays of the patient's spine. The arrows indicate the vertebral fracture (L2)



In recent years however, and on the basis of descriptions in the literature of cases of osteonecrosis of the jaw (ONJ) and atypical femoral fractures in patients being treated with bisphosphonates[5,6], a strand of opinion is gaining strength towards ceasing treatment solely because of the fact that "it has carried on for some considerable time", "because there are no data on its safety after a certain number of years" or "due the risk of the appearance of the same type complication as osteonecrosis of the jaw or atypical femoral fracture". It seems to us that these justifications don't carry much real weight. In terms of duration of treatment, perhaps we can raise this question in the treatment of other chronic diseases such as AHT or diabetes. A pathology, while it exists, should be treated, *a priori*, and in the absence of complications or secondary effects, the treatment should be maintained with the drug of choice if it is being effective.

On the other hand, the bisphosphonates are quite safe drugs whose single demonstrable adverse effect is a lesion in the oesophageal mucosa, which is avoided through postural measures after its oral administration. It is true that there are no reliable safety data for such a long period of treatment with bisphosphonates. However, it is difficult for these to be collected (at least not within the terms of a clinical trial, with a high number of patients, randomised, controlled and double blind to ensure reliability) because clinical trials in the

Table 1. Biochemical data related to bone mineral metabolism. 25-HCC: 25-hydroxycalciferol. TRAP: tartrate resistant acid phosphatase. P1NP: amino-terminal propeptide of procollagen type 1. CTX: carboxy-terminal telopeptide of collagen type 1. NA: not applicable

Parameter	Reference range	2004	2014	Percentage change compared to baseline
25-HCC (ng/mL)	30.0 - 80.0	22	51.8	57.5%
PTH (pg/mL)	15 - 88	54.2	29.9	-81.2%
Calcium (mg/dL)	8.5 - 10.5	8.9	9.3	4.3%
Phosphorus (mg/dL)	2.5 - 4.9	3.2	3.2	0%
Osteocalcin (ng/mL)	11 - 43	8.6	13.45	55.8%
TRAP (UI/L)	0.0 - 3.3	2.4	2.9	17.2%
P1NP (ng/mL)	Premenopausal: <30.1 Postmenopausal: <37.1 Men: <36.4	NA	22.54	NA
CTX (ng/mL)	0 - 0.5	0.4	0.15	-166.6%
Corrected calcium (mg/dL)	8.5 - 10.5	9	9.4	3.1%

field of osteoporosis only last 3 to 5 years. Once approval for the drug's use is given the study concludes. There are very few long term follow up studies, and in these the loss of numbers of patients is so high that it begins to raise questions about whether the remaining population is representative of that at the start of the study. Therefore, once a drug comes onto the market it is the clinical experience which matters. Then, if there are no adverse effects, and the drug continues to be effective, the clinical experience has nothing to say against the continuation of the treatment.

Table 2. Estimation of the risk of fracture at 10 years at the first visit

Estimated absolute risk	FRAX®	QFRACTURE®
Any fracture *(Major)*	15	6
Hip fracture *(hip)*	2.7	2

The feared secondary effects associated with the long term treatment with bisphosphonates, attributed to its powerful antiresorptive effect, such as osteonecrosis of the jaw or atypical fractures, are not a real problem. Osteonecrosis of the jaw associated with bisphosphonates is a complication which mainly appears in cancer patients treated with these drugs for their bone metastases, who, in addition, have received other powerful treatments (cytostatics), and in whom the bisphosphonates are used at doses much higher than those used in the treatment of osteoporosis[5]. On the other hand, while the precise etiopathology of ONJ is not known, these days it has already been identified as having a multifactorial etiopathology, which would include factors not only related to the treatment received by the patient (the bisphosphonates certainly, but also the glucocorticoids), but also the presence of concomitant diseases (such as diabetes mellitus or rheumatoid arthritis), as well as the concurrence of dental intervention (extraction, implants, etc.), accompanied by an infectious component[7]. Yet, even if this were not enough for ONJ not to be considered as a real problem in the treatment of OP with bisphosphonates, it is known that up to 25% of the cases of ONJ reported, bisphosphonates were not taken[5,8]. In those cases described in osteoporotic patients treated with bisphosphonates, studies of its incidence talk in figures of around 1/100,000 patients/year and even less than 1/100,000 patients/year[9]. In the reference study of zoledronate (HORIZON), which considered ONJ as an adverse effect, the appearance of only two cases were confirmed, one of which occurred in the placebo group[10]. A systematic review, which evaluated whether patients in treatment with bisphosphonates, both I.V. and orally, had a greater risk of suffering ONJ before the performance of a dental implant than those not being treated with this

Table 3. Densitometry values over 10 years of development. BMD: bone mineral density

Year	BMD L2-L4	T-score L2-L4	BMD femoral neck	T-score femoral neck	BMD total hip	T-score total hip
2004	0.655	-3.7	0.607	-2.1	0.778	-1.3
2005	0.717	-3.1	0.639	-1.8	0.845	-0.8
2006	0.734	-2.9	0.648	-1.8	0.850	-0.8
2007	0.765	-2.6	0.638	-1.8	0.843	-0.8
2008	0.744	-2.8	0.671	-1.5	0.852	-0.7
2010	0.744	-2.8	0.638	-1.8	0.825	-1.0
2011	0.757	-2.7	0.647	-1.8	0.844	-0.8
2012	0.777	-2.5	0.646	-1.8	0.870	-0.6
2014	0.776	-2.5	0.673	-1.5	0.884	-0.5

drug, concluded that treatment with bisphosphonates was not a contraindication for carrying out this intervention[11]. Increasing numbers of researchers are concluding that ONJ is such an infrequent complication in patients treated with bisphosphonates for osteoporosis that its risk does not justify cessation of long term treatment, and more so when this is a treatment which effectively reduces the risk of fracture, a complication whose incidence is incomparably higher than that of ONJ, as well as its morbidity, mortality and socioeconomic cost.

On the other hand, atypical femoral fractures are currently the principal argument in favour of the suppression of treatment with bisphosphonates, since in various epidemiological studies it has been established that there is a definite association between the risk of developing an atypical fracture and the period of time over which bisphosphonates are used[12]. However, it is also certain that cases of this type of fracture have been described in patients who are not taking bisphosphonates, but with other antiresorptive drugs such as denosumab[13], and even others not used for osteoporosis, such as proton pump inhibitors and the glucocorticoids. It has also been associated with other pathological conditions, such as hypophosphatasia, vitamin D deficit and rheumatoid arthritis[14]. Overall, the risk of atypical femoral fracture is very low. A study carried out by Black et al. which analysed femoral fractures which occurred during three clinical trials (FIT, FLEX, and HORIZON, carried out with alendronate and zoledronate), found that in a total of 14,194 women included in the study there were 283 femoral fractures, of which only 12 were atypical[15]. Its incidence has been estimated at 32 cases/million patients/year[12] or 1.78/100,000 patients/year[16], and even though the same studies found an increase in incidence with years of treatment –of 10%/year[15] and 113.1/1000,000 patients/year[16]– even this incidence is not sufficient to affect the risk/benefit ratio of these drugs. With the evidence currently available it is not possible to establish a causal relationship between prolonged treatment with bisphosphonates and the appearance of atypical fractures, it being probable that these drugs play a role in their development, but not possible that they provide the sole condition for the development of these fractures[17].

The final reason which might justify the discontinuation of treatment is the observation that the biphosphonates have a certain residual effect and that once stopped the reduction in the risk of fractures is prolonged without the continuation of the drug. The reference study for alendronate, called the FIT study, confirmed that after a follow up of an average of 4.3 years, patients who took alendronate had a reduction in the risk of morphometric vertebral fractures of 47%, clinical fractures of 55% and hip fractures 51%[18].

The researchers in this study extended it a further 5 years, calling it the FLEX study, comparing the reduction in risk of fracture between those who continued to take alendronate and those who stopped taking it. It was observed that when the treatment was stopped there was a residual effect on the reduction in risk of non-vertebral fracture, but in contrast, the risk of vertebral fracture increased in comparison with those patients who had continued taking the drug, in whom the reduction in the risk of vertebral fractures was 55%[19].

But not even taking the drug correctly gave the patients 100% protection. Or, stated in another way, the risk never reduced to 0%. However, there is an additional risk factor which is highly significant, and inescapable, namely age. In our patient, only taking the age factor into account, her risk of

fracture has increased because she is 10 years older. If we now suggest the patient has "treatment holidays" (a euphemism for cessation of treatment) we are ignoring the increased risk due solely to the fact that she is 10 years older. What, then, is the aim of initiating treatment for osteoporosis? To avoid the occurrence of fractures, if possible throughout the patient's life? Or to delay their appearance until 10 years later?

In this patient the treatment for osteoporosis has been effective to date. There have been no secondary effects or complications of any kind. The biochemical markers for bone remodelling are normal, there is no "oversuppression" of bone remodelling. The BMD is increasing. And the patient is 10 years older. Solely for this reason her risk of new fractures is now even greater. If we stop the patient's treatment (that is, give her treatment holidays) simply because she has been taking it for 10 years, it is possible that the protection achieved would be reduced to the point at which it no longer counteracts the increased risk of fracture which is due to the same fact, the patient being 10 years older.

In our opinion, therefore, stopping treatment for this patient is neither necessary nor advisable.

B) Reasons for carrying out treatment holidays in this patient (YES to treatment holidays)

Jorge Malouf Sierra

As has been stated earlier, the aim of any treatment for osteoporosis is to reduce the risk of fragility fractures. These types of fracture appear when the resistance of bone is not capable of maintaining the integrity of bone tissue and the most innocuous biomechanical forces provoke a collapse in bone structure.

The NICE guides for the assessment and treatment of fragility fractures 20 suggest that an assessment of the risk of fracture should be made in women below 65 years of age, only if they have other associated risk factors, among which may be:

- Previous fragility fracture.
- Current use of glucocorticoids.
- History of fractures.
- Family history of femoral fractures.
- Secondary causes of osteoporosis.
- Low body mass index (lower than 18.5 kg/m^2).
- Tobacco use.
- Consumption of more than 14 units of alcohol per week.

In the case of our patient, it may be considered appropriate to assess the risk of fracture by means of DXA, given that she has a vertebral fracture. However, she has only one such fracture. Ismael et al. reported in 2001 that vertebral fractures are a predictive factor for subsequent vertebral fractures, as well as hip and non-vertebral fractures. However, the increase in risk was significant only after two or more vertebral fractures[21].

Also, the patient's clinical history does not confirm whether this fracture was related to any trauma, or what degree of fracture it was. It is reported that the number and severity of fracture(s) increases the risk of fractures[22], independently of the patient's BMD. Even so, the BMD which best predicts the risk of fracture is that in the femoral neck.

Figure 2. Change in bone mineral density in the spine

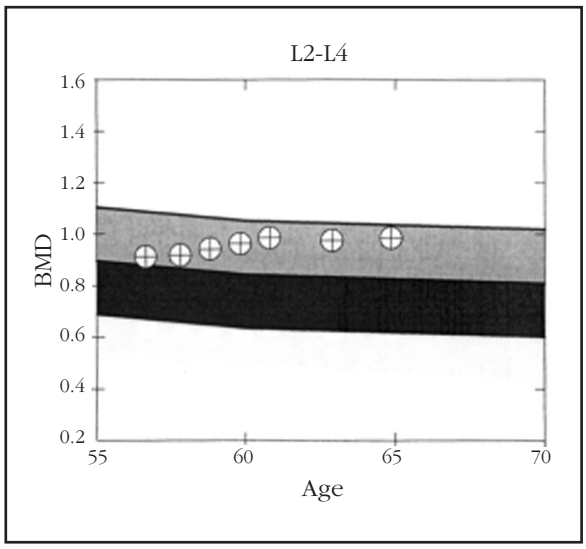
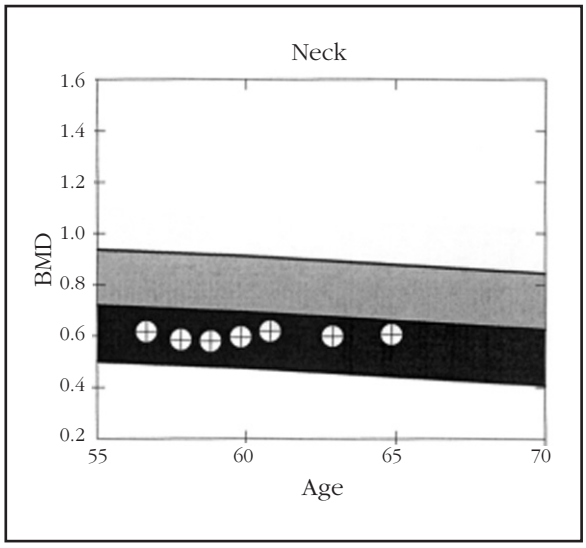


Figure 3. Change in bone mineral density in the femoral neck



Returning to the clinical case: during 10 years of follow up and periodic densitometries the patient never had a BMD in the femoral neck compatible with osteoporosis, this being a T-score lower than -2.1 at the start of the development of this pathology.

As is also mentioned earlier, one of the most significant risk factors for fractures is age. At the start of treatment, when the patient was 53 years of age, the risk of fracture was very low, and although the BMD in the lumbar spine was low, there was not a very high risk of fracture – vertebral or hip.

The bisphosphonates are antiresorptives which have a great affinity with bone tissue. Patients who receive a bisphosphonate over long periods will have bisphosphonate bonded with the bone for a long time. This has meant that, in recent years, various adverse effects have appeared, which are usually observed after a period of treatment. These adverse effects are atypical hip fracture and bisphosphonate-related osteonecrosis of the jaw (ONJ). The latter was reported for the first time by Marx in 2003[23], but to date, although it is known that there is a relationship between the prolonged use of bisphosphonates and this pathology, the strength of this relationship is not clear. It is known that there are various risk factors which increase the probability of suffering ONJ, such as the duration of treatment, genetic factors and demographic factors, such as age, among others[24].

The patient in the clinical case is a woman of over 60 years of age who has been receiving oral bisphosphonates for 10 years, which increases the risk of the appearance of ONJ. On the other hand, the patient's long period of treatment with bisphosphonates also provides some advantages, such as the fact that the bisphosphonates continue to be released by the bone over a long period of time, reducing the risk of fracture in spite of the patient not continuing with the treatment. This strategy is known as a treatment holiday[25].

The longest placebo controlled study there has been was with risedronate, and the results regarding its efficacy and safety come after 5 years of treatment. This trial assessed the reduction in risk of fracture and demonstrated an additional effect during the final years of treatment. During the following year or two the markers for bone remodelling changed little. Although there is no clinical trial which demonstrates that the risk of fracture stays low during this "treatment holiday" period, it may be assumed that, if the markers for bone remodelling don't change, the patient with a low risk of fracture may be protected during this time and it would not be necessary for them to continue to take bisphosphonates 26. Similarly, Black et al. showed that continuing treatment with zoledronic acid for 6 to 9 years did not bring any benefits in respect of a reduction in the risk of fracture[4].

Finally, the Spanish Society for Bone and Mineral Metabolism Research (SEIOMM) has published a document in which it recommends that after 5 years of treatment with oral bisphosphonates all patients be evaluated with aim of assessing the risk of fracture of the particular patient, and thus to decide whether to continue with the bisphosphonate (in cases where the risk is high) or discontinue treatment. In addition, it recommends that in those patients with a T-score above -2.5 in the femoral neck temporary cessation of treatment (treatment holidays) be considered[27].

So, in the case of this patient, the only risk factors which remain are age and history of vertebral fracture. During 10 years of treatment the BMD of the patient has developed satisfactorily, currently being borderline in the spine (T-score: -2.5) and at osteopenic levels (T-score: -1.5) in the femoral neck. These data suggest that the patient should not continue with the treatment with oral bisphosphonates and should have treatment holidays. Later, the patient's risk of fracture should be assessed annually, investigating specifically her BMD, changes in levels of markers for bone remodelling and paying most attention to the progress of her pre-existing vertebral fracture or the production of new vertebral fractures, in which case she should be reassessed for the initiation of treatment, be it with antiresorptive or osteoforming drugs.

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