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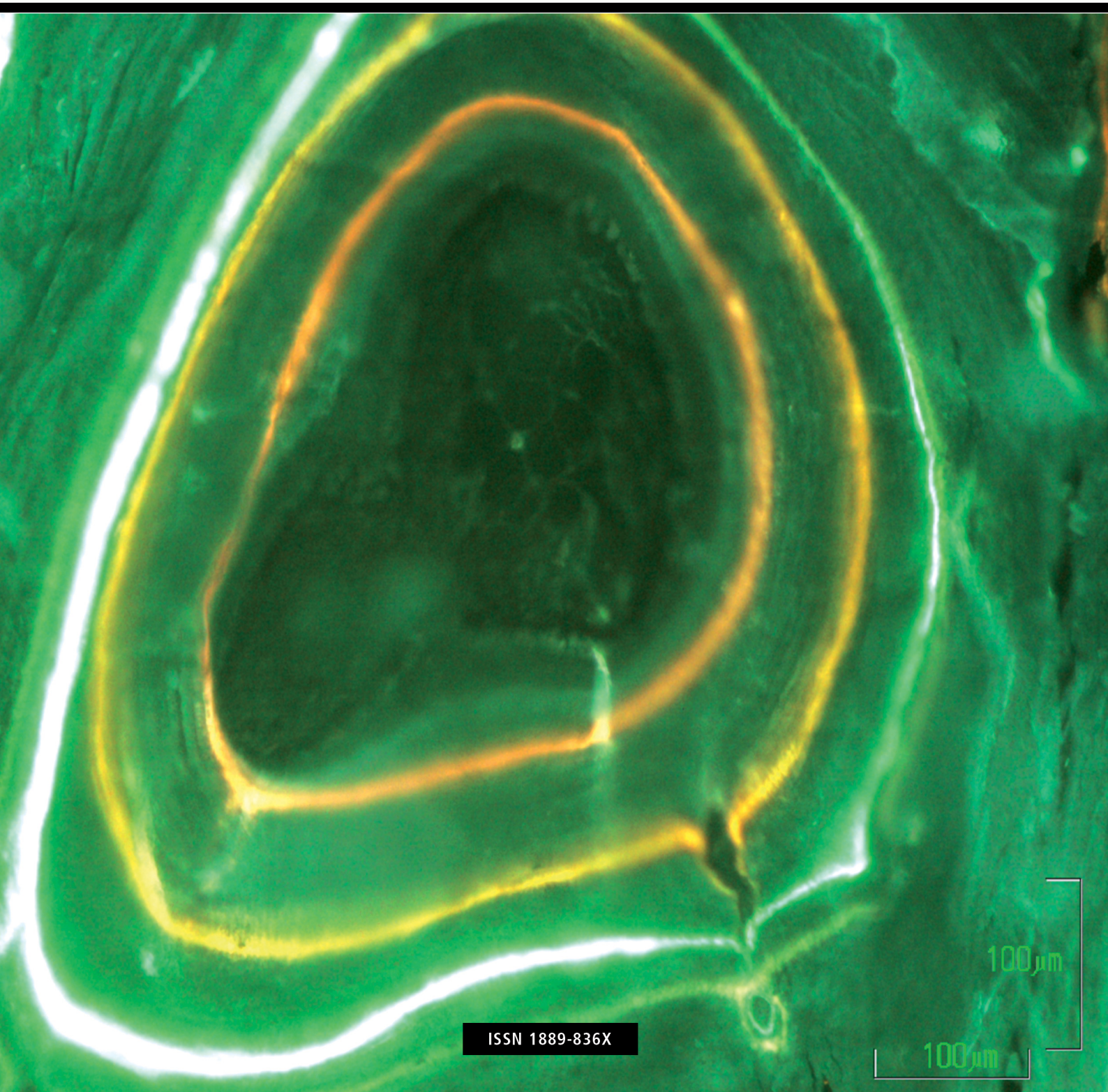

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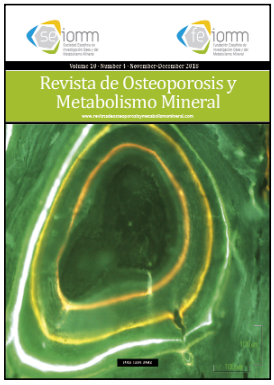

Our cover
Bone marking with fluorescence

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SUMMARY

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Atypical femoral fractures: a rare complication possibly due to the accumulation of rare genetic variants

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Antiresorptive drugs, such as bisphosphonates and denosumab, are very effective in reducing the risk of vertebral and non-vertebral fractures in patients with osteoporosis. They can be administered conveniently, are generally well tolerated and the side effects are mild and infrequent. Occasionally, however, some patients may present complications peculiar to the treatment, such as atypical femoral fractures (FFA) and maxillary osteonecrosis. These complications occur very rarely, but are potentially serious and difficult to manage, so they are a source of concern for some doctors and many patients. This fear seems to have a negative influence, although not justified, on therapeutic compliance. Therefore, it would be extremely useful to identify the rare patients who are at risk of developing these complications.

FFA is a particularly paradoxical case, since it involves fractures that appear associated with treatments that are given precisely to reduce the risk of fracture. The ASBMR (American Society for Bone and Mineral Research) has developed criteria to identify atypical fractures, which include a subtrochanteric or diaphyseal location, an origin in the outer cortex and a transverse or slightly oblique path, a minimal or absent comminution, a thickening Periosteal in the external cortex and the absence of high-impact trauma as a trigger[1]. FFA has been related mainly to bisphosphonates, but cases associated with other antiresorptive drugs have also been reported[2]. Likewise, the appearance of fractures with characteristics similar to FFA has been described in patients with some monogenic skeletal diseases, such as osteogenesis imperfecta, pycnodysostosis, osteopetrosis, hypophosphatemic rickets or hypophosphatasia, even without having received antiresorptive drugs[3,4].

The frequency of FFA varies markedly from one study to another. Estimates range from 3 to 50 cases per 100,000 patient-years of bisphosphonate treatment. Prolonged treatment, for more than 5 years, seems to be associated with an increased risk, with the incidence reaching, in these cases, about 130 cases per 100,000 patient-years[5]. In several studies, clinical factors associated with FFA have been explored. Among them, treatment with glucocorticoids together with bisphosphonates is the one that has been associated with an increased risk of FFA in a more consistent manner[5,6].

As FFA is a complication that appears very rarely, only in a minority of patients treated with antiresorptives, it is thought that individual predisposition must be a significant factor. In favor of this is the fact that with a certain frequency the fractures are bilateral in the affected patients. Hence, several authors have analyzed whether these patients have any predisposing genetic characteristics. In this line, the work of Roca-Ayats et al.,[7] published in this issue of the journal, is particularly interesting because it includes three sisters with FFA. The family association reinforces the notion of genetic predisposition. The authors sequenced the exome, that is, the DNA coding regions. Most inherited diseases are due to mutations in these regions, although they only account for about 1% of the DNA. Roca-Ayats et al. They found several mutations in the 3 sisters studied, including some in genes that encode enzymes of the mevalonate pathway. These mutations are particularly interesting because this pathway is target of bisphosphonates, which gives biological plausibility to the causal relationship between these variants and the FFA associated with these antiresorptive drugs. However, the authors could not confirm that these mutations were involved in FFA suffered by other patients unrelated to the previous ones. In the Roca-Ayats study it was also observed that some patients had a mutation in the CYP1A1 gene, which metabolizes various hormones, eicosanoids and exogenous agents. Other studies have found mutations in genes that encode bone proteins such as alkaline phosphatase or collagen in some patients isolated with FFA. But in most of the cases analyzed, these mutations were not found[4].

These results suggest that there is genetic heterogeneity, that is, the susceptibility genes vary from one patient to another. *In silico* analyses and some functional experiments suggest that these muta-

tions have a deleterious effect on the function of proteins[8]. However, it must be taken into account that mutations have not yet been shown to be directly related to FFA risk.

Another issue that is not definitively clarified is whether FFAs respond to a monogenic or polygenic pattern, that is, if they are determined by a single variant in a given gene (although different from one patient to another) that causes a serious defect in bone biology, or if they are due to the accumulation of variants with negative effects in several genes, each of them with a limited impact. In a previous study of genotyping of patients with FFA using an exon-chip technology, which analyzes rare variants in the exome, we found that patients tended to accumulate variants not present in control subjects[9]. This supports the idea of a polygenic susceptibility. However, these results have yet to be confirmed in other groups of patients.

Although the results published in this field are still very few, the absence of replication is striking. That is, the genetic variants associated with FFA, a) are different in the different studies, and b) differ among the different patients in the same study. Logically, the work of Roca-Ayats is an exception in this last aspect, since it included several members of the same family. This suggests that the variants that predispose to FFA are rare variants, very rare in the general population, probably typical of a specific population group, or even of a specific patient. If this is really the case, it will be very difficult to replicate the results in different populations.

In fact, some epidemiological studies support the importance of genetic background and race in susceptibility to FFA. Thus, this complication seems to be much more frequent among Asians than in the Caucasian population[5,10]. On the other hand, FFA may be favored by certain characteristics of skeletal development. In fact, several studies have found an association between the curvature of the femur and FFA, so that FFA would be more frequent in patients with a varus femur[11]. But this phenomenon is not universal. Some patients with FFA do not present varus of the femur and in them the susceptibility presumably is conditioned by anomalies of the remodeling or other alterations of the bone biology, more than by alterations in their geometry.

The studies of genomic scanning and exome analysis are providing the first data to shed light on the determinants of individual susceptibility to FFA. To advance in this field, on the one hand, genetic studies of much larger groups of patients are needed. On the other hand, functional studies that demonstrate the real impact of these genetic variants on bone, through the analysis of transgenic and knock-out animals and other gene editing experiments. But keep in mind that it will not be enough to analyze the skeleton of genetically modified animals under basal conditions, but it will also be necessary to determine the skeletal changes in response to the antiresorptive.

There are other aspects not yet explored and whose involvement in the FFA cannot be ruled out *a priori*. These include, for example, alterations in DNA regulatory regions (non-coding regions not included in the exome analysis) and epigenetic marks such as DNA methylation and post-translational modifications of histones.

In short, the published clinical studies suggest that there is an individual susceptibility to FFA, determined, at least in part, by genetic factors. Such aspects have not yet been identified with certainty, but they may be polygenic, related to the accumulation of rare mutations in diverse genes. The Roca-Ayats study is a very interesting contribution to a question that has still hardly been explored. In anticipation of advances in this field, which should ideally lead us to be able to identify patients at risk early, clinicians and patients should not forget that FFAs are much less frequent than fragility fractures and that the risk-benefit ratio of antiresorptive drugs is clearly favorable. It has been estimated that for every FFA that could appear related to antiresorptive treatment, more than 100 hip fractures and several hundred other fractures are prevented[12]. Therefore, a very infrequent adverse effect such as FFA should not be an impediment for patients with osteoporosis to receive antiresorptive treatment when indicated and thus benefit from the marked reduction in fracture risk achieved with these drugs.

Conflict of interests: José Antonio Riancho has received research scholarships, conference fees or travel allowances from MSD, Alexion, Lilly, Nycomed and Amgen.

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Genetic study of atypical femoral fractures using exome sequencing in three affected sisters and three unrelated patients

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Summary

Objectives: Atypical femoral fractures (AFF) are rare, often related to long-term bisphosphonate (BPs) treatment. Their pathogenic mechanisms are not precisely known and there is no evidence to identify patients with a high risk of AFF. The aim of this work is to study the genetic bases of AFFs.

Material and methods: Whole-exome sequencing was carried out on 3 sisters and 3 unrelated additional patients, all treated with BPs for more than 5 years. Low frequency, potentially pathogenic variants shared by the 3 sisters, were selected, were selected and a network of gene and protein interactions was constructed with the data found.

Results: We identified 37 rare variants (in 34 genes) shared by the 3 sisters, some not previously described. The most striking variant was the p.Asp188Tyr mutation in the enzyme geranylgeranyl pyrophosphate synthase (encoded by the *GGPS1* gene), from the mevalonate pathway and essential for osteoclast function. Another noteworthy finding was two mutations (one in the 3 sisters and one in an unrelated patient) in the *CYP1A1* gene, involved in the metabolism of steroids. We identified other variants that could also be involved in the susceptibility to AFFs or in the underlying osteoporotic phenotype, such as those present in the *SYDE2, NGEF, COG4* and *FN1* genes.

Conclusions: Our data are compatible with a model where the accumulation of susceptibility variants could participate in the genetic basis of AFFs.

Key words: *atypical femoral fractures, bisphosphonates, GGPS1, CYP1A1, whole-exome sequencing.*

Introduction

Osteoporosis and its associated fractures are the most common postmenopausal bone problems, affecting women and men of all ethnic groups. Nitrogen-containing bisphosphonates (N-BPs), including alendronate, risendronate, ibandronate and zolendronate figure as the most widely used osteoporosis treatments in millions of patients worldwide. Despite the significant anti-fracture efficacy of BPs, which has been widely demonstrated in several clinical trials[1] and systematic reviews[2], some infrequent adverse effects associated with prolonged use have been described, including atypical femur fractures (AFFs)[3]. These fractures are non-traumatic and characterized by their subtrochanteric location or in the diaphysis of the femur, and are frequently bilateral[4].

AFFs' pathogenic mechanisms are not completely known and much has been speculated about their causes. An excessive suppression of bone resorption by N-BPs could trigger an AFF but its pathophysiology is complex and other important factors are reportedly involved. Some proposed risk factors are cortical thickness and pelvic geometry[5]. In addition, cases of AFF have been described in patients affected by other monogenic bone diseases, such as hypophosphatasia[6], osteogenesis imperfecta[7] or the syndrome of osteoporosis pseudoglioma[8].

Given the low incidence of AFFs in the general population (5.9 cases per 100,000 people/year), we can hypothesize that there are underlying rare genetic causes that may increase susceptibility to AFFs, and that they might occur spontaneously or be triggered after the interaction with the BPs. Currently, there are no genetic or biochemical tests to help identify patients with a high risk of AFF. Identifying the genetic determinants of AFFs would help to clarify the etiological mechanisms, develop diagnostic tools and evaluate AFF risk and possible therapeutic strategies.

Previously, we identified 3 sisters diagnosed with AFF who were treated with BPs for more than 5 years[9]. This observation suggested that there might be a genetic background predisposing to AFFs related to prolonged use of BPs. Consequently, we carried out the sequencing of the complete exome of the 3 sisters and of 3 other unrelated patients to identify mutations potentially related to the AFFs in these patients. We identified 37 rare variants shared by the 3 sisters, one of which was studied in detail[9]. In the present work, we describe the set of variants found and their possible interaction.

Material and methods

Patients

Six patients with AFFs who had been treated for more than 5 years with BPs were studied: 3 sisters visited at the Reina Sofía University Hospital (Córdoba, Spain) and 3 unrelated patients visited at the Hospital del Mar (Barcelona, Spain). As controls, 3 patients treated with BPs for more than 6 years but without AFFs were studied. The characteristics of patients and controls are described in Table 1. The 3 affected sisters were treated with statins and received PPIs regularly but had not been treated with glucocorticoids or any other compound that affects the bone, apart from the BPs. In the case of unilateral fractures, radiological and MRI tests were performed that ruled out the contralateral fracture. Written informed consent was obtained from all patients, in accordance with the regulations of the Clinical Research Ethics Committee of the Mar Health Park, which approved the study.

Complete exome sequencing

Peripheral blood DNA was extracted from the patients with the Wizard Genomic DNA Purification kit (Promega) and used to sequence the complete exome at the National Center for Genomic Analysis (CNAG) (Barcelona). Libraries were generated with the SureSelect XT Human All Exon exons capture kit; cat: 5190-6208 (Agilent Technologies), after having fragmented the DNA and ligated the specific Agilent adapters. Paired-end sequencing (2x76 bp) was carried out on the Illumina HiSeq2000 platform. The images were processed using the manufacturer's program to generate FASTQ sequence files.

The bioinformatic analysis was carried out in the Bioinformatics platform for Rare Diseases (Bier) of the CIBERER, in Valencia. The FASTQ files were aligned with the free program Burrows-Wheeler Aligner[10] (http://bio-bwa.sourceforge.net/) using the reference human genome assembly GRCh37 (hg19)[11]. Single-nucleotide and indel variants were identified using the GATK program[12]. Finally, to add to the variants information on the frequency of the minority allele (MAF) from dbSNP and the 1000 Genomes project (http://www.1000genomes.org)[13], the annotation tool VARIANT was used[14]. The data were converted to the BAM (binary equivalent SAM) format and visualized using the Integrative Genomics Viewer (IGV) program (http://www.Broadinstitute.org/igv).

The genetic variants were filtered according to the following premises: a) non-synonymous variant, b) not previously described or with an MAF <0.005 in dbSNP and in the 1000 Genomes project, c) not present in the NHLBI Go Exome Sequencing Project (ESP) (http://evs.gs.washington.eu/EVS/), and d) not present in 8 exomes of individuals from the general population, obtained in our laboratory.

Initially, only the mutations shared by the three sisters were considered, both in a model of dominant and recessive inheritance. Next, mutations in candidate genes were prioritized in the other three patients. The SIFT[15], PolyPhen[16] and evolutionary conservation scores obtained from PhastCons[17] were used to prioritize the variants.

Validation of genetic variants

The mutations found were validated by PCR and Sanger sequencing, which was carried out bidirectionally using the BigDyeTM v3.1 Terminator Cycle Sequencing kit (Applied Biosystems), according to the manufacturer's instructions. The pri-

mers used for the validation were designed using the OligoEvaluator program (Sigma-Aldrich). Finally, the validated mutations were searched in the Exome Aggregation Consortium (ExAC) to obtain their population frequencies, and analyzed by Sanger sequencing in the 3 control women.

In silico analysis

Mutations were localized in their genetic context using the UCSC Genome Browser (https://genome.ucsc.edu/) and the Ensembl Genome Browser (http://www.ensembl.org/) and extracted information from GeneCards genes (http://www.gencards.org/) and BioGPS (http://biogps.org/). A functional enrichment analysis was carried out using the bioinformatics tool DAVID[18] (https: //david. Ncifcrf.gov/).

In silico functional study of the mutated proteins was carried out using Uniprot (http://uniprot.org), RCSB Protein Data Bank (PDB) (http:// www.rcsb.org/pdb) and Pfam (http:// pfam.xfam.org). The protein alignments were made using the UCSC Genome Browser and the Clustal Omega programs (http://www.clustal.org/omega) and ESPript (http://espript.ibcp.fr).

Construction of the network

The AFF gene interaction network (AFFGeNet) was constructed according to Boloc *et al.*[19] to identify genes or proteins that interact with the 37 FAF genes, considered as driver genes (Tables 2a and 2b), taking into account the binary and directional interactions. The high-throughput interaction data were obtained from BioGRID (version 3.4.133)[20] and STRING [Search Tool for the Retrieval of Interacting Genes/Proteins] version 10[21] and the network was enriched with additional information from GeneOntology (http://geneontology.org), GeneCards, OMIM, UniProt, RefSeq, and UCSC.

A Perl script was implemented to capture the interaction sub-network using the AFF genes to find all the shortest paths between two genes by applying the Dijkstra algorithm. The connectivity in pairs was analyzed using Circos[22]. The script produced a skeleton graphic in JSON format in order to visualize the data in the AFFGeNet web interface (https://compgen.bio.ub.edu/AFFgenes, available on demand). The web form contains an entry that focuses on the selected genes, and the visualization of the network allows you to add or remove nodes and display information of the AFF genes. The border color identifies the nodes as drivers (lilac), upstream (green) or downstream (blue) pairs of the selected drivers, and others (gray). The color of the interior of the nodes represents the specific gene expression of the bone, which was obtained from the Gene Expression Omnibus (GEO)[23], specifically from a study on osteoclast precursor cells treated or not treated with BPs (alendronate or risendronate) during their differentiation to mature osteoclast[24] (GSE63009). The color scale goes from intense yellow (underexpressed) to dark blue (overexpressed), being the target indicative of no change of expression.

Results

Variants detected in the sequencing of the complete exome in the 3 sisters

The three sisters (AFS1, AFS2, AFS3) and the 3 unrelated patients (AFU1, AFU2, AFU3) were analyzed separately.

Table 1. Characteristics of patients and controls

Patient	Fracture atypical	Age[a] (years)	Weight (Kg)	T-score spine	T-score hip	Time of treatment with BPs (years)	Fractures previous osteoporotic
AFS1	Unilateral; half-diaphyseal[b]	64	77	-1.1	-0.2	6	Colles
AFS2	Unilateral; half-diaphyseal[b]	73	75	-2.5	-1.4	6	Colles
AFS3	Bilateral; half-diaphyseal[b]	60/61	100	-0.3	Rbpc[c]	6	Any
AFU1	Bilateral; half-diaphyseal	73/75	50.8	-1.9	-0.5	6	Any
AFU2	Unilateral; half-diaphyseal	72	90	-2.0	-0.6	7	Any
AFU3	Unilateral; subtrochanteric	87	59.8	N/A	N/A	10	Any
Control 1		78	66.5	-2.5	-1.9	7	Any
Control 2		70	57.5	-1.2	-2.4	6	Any
Control 3		74	77.1	-1.5	-0.9	8	Any

AFS: sisters with AFF; AFU: patients with unrelated AFF, ([a]): age at the time of atypical fracture; ([b]): fractures located approximately at the same site; ([c]): bilateral replacement of hip prosthesis.

The exomes of the 3 sisters intersected and no variant in common homozygosis was identified. On the contrary, 74 variants were identified in shared heterozygosis (consistent with a dominant inheritance model), 37 of which were validated by Sanger sequencing. In 3 of the genes (*FN1, BRAT1* and *XAB2*), 2 different mutations were found. In all three cases it was possible to determine that the variants were in phase, being double-mutant alleles and non-heterozygous compounds, by visualizing the reads with the IGV program and the analysis of intragenic polymorphisms. The 37 variants shared by the 3 sisters, all of them coding, are shown in table 2a, ordered according to their conservation score. These are change-of-sense variants (n=35), a truncating variant and a phase deletion. The first variant of the list, with the best conservation score and predicted as deleterious, is found in the *GGPS1* gene, as described above[9].

Analysis of mutated genes in the 3 unrelated patients

The genes with variants shared by the 3 sisters (Table 2a) were analyzed in the exomes of the unrelated patients using the IGV program. None of the variants of Table 2a was found in unrelated patients. However, two other variants were found in the *BRAT1* and *CYP1A1* genes, in patients AFU3 and AFU1, respectively (Table 2b).

The variant of *CYP1A1* present in the patient AFU1 (p.Ser216Cys) involves the change of a serine to a cysteine, in a position close to the site of binding to the substrate. Predictors of pathogenicity suggested that this change is very deleterious for protein function. Similarly, the variant *CYP1A1* present in the three sisters (p.Arg98Trp) involves the change of a basic amino acid (arginine) to a hydrophobic aromatic amino acid (tryptophan), in a protein spin with hydrogen bonds. In contrast, the three variants found in the *BRAT1* gene (two in the three sisters, in a mutant double allele, and one in the patient AFU3) do not affect the function of the protein, according to the predictors.

Analysis of candidate genes in 3 unrelated patients

Next, the IGV program was used to analyze, in the exomes of the three unrelated patients, different genes involved in bone metabolism, osteoclastic function and the mevalonate pathway. Variants were found in the *MMP9* (AFU3), *MVD* (AFU2) and *RUNX2* (AFU3) genes, which were validated by Sanger sequencing (Table 2b). The mutation in the *MMP9* gene, which encodes type IV collagenase, involves the change of a methionine (a hydrophobic amino acid with a sulfur-containing group) to a threonine (hydrophilic amino acid) at position 419, within the catalytic domain. This variant appears in the ExAC database, with a very low allelic frequency (8.2e-06), and SIFT and PolyPhen predicted that it probably impairs its function. The *MVD* gene encodes the enzyme mevalonate 5-diphosphate decarboxylase, from the mevalonate pathway. The variant found (p.Arg97Gln; rs376949804) involves the change from a basic amino acid to a neutral amino acid and is present in the ExAC database, also with a very low allelic frequency (3,4e-05). It is a change not harmful to the function of the protein, according to SIFT and PolyPhen. The mutation in RUNX2 is a substitution of a proline, a cyclic amino acid, by a leucine, a hydrophobic aliphatic amino acid, at position 296, within a region rich in prolines, serines and threonines. This change, described in dbSNP (rs20184115), has an MAF=0.0004 and probably affects the function of the protein, according to the predictors.

Analysis of the variants in control individuals and in the general population

No variant of tables 2a and 2b was found in 3 controls (patients treated with BPs for a long period of time but without AFFs). All the variants detected in patients with AFF were searched in the ExAC database to determine if they were new or very rare variants (MAF <0.005). In this sense, eleven mutations were found neither in dbSNP nor in ExAC (*GGPS1*: p.D188Y, *COG4*: p.G85D, *PGRMC1*: p.P177H, *TMEM25*: p.V239del, *HEPHL1*: p.W991*; *CUL9*: p .T423I; *IQCF6*: p.R61W; *MGA*: p.S571L; *SHC4*: p.H180N; *SMS*: p.G14R; *BRAT1*: p.E458L). The other variants have frequencies ≤1/10000, according to ExAC.

Network of gene/protein interaction and path enrichment

A network of interactions between genes and/or proteins was constructed to investigate the functional pathways related to the 37 mutated genes found in the sequencing of the exomes and detect other potentially causative genes, as well as molecular mechanisms that may be involved in the generation of the genes. AFFs. Figure 1 shows the connectivity between gene pairs. In different circles, the input and output connections for the 37 genes are shown at distances 1 to 4, respectively. At distance 1 there are almost no interactions, with *FN1* being the only gene connected to others. At distance 2 more connectivity is observed. The majority of the connectivity between pairs of genes is observed at a distance. The only gene that does not present any interaction at any level is *IQCF6*.

The network of gene/protein interactions shows that *GGPS1* and *CYP1A1*, two of the most relevant driver genes, are connected at distance 3, through *INS* and *IL6* (Figure 2a). Another 4 driver genes (*RUNX2, MVD, MMP9* and *PGRMC1*) are connected to *GGPS1* at distance 2. *MMP9* is also remote 2 of *CYP1A1*. In addition, *FN1* and *MMP9* are connected remotely 1. Similarly, the driver genes *SYDE2* and *NGEF* are interconnected at distance 2, via *RHOB* (Figure 2b).

The path enrichment analysis in the 37 mutated genes, carried out with the DAVID tool, resulted in the isoprenoid biosynthesis pathway (GO: 0008299) (p=0.0006), which contains the *GGPS1*, *MVD* and *CYP1A1* genes.

Discussion

In this work, we have studied the genetic background of 3 sisters with AFF and 3 additional patients, unrelated, through the massive sequencing of the exome to identify possible susceptibility genes to the pathology. We have identified 37 rare variants (in 34 genes) shared by the 3 sisters, some of them not previously described and considered harmful by the predictors. The most striking variant was the mutation p.Asp188Tyr in the *GGPS1* gene, which presented the best conservation score, and which we have already described in a previous work[9]. Another interesting finding was the two mutations in the *CYP1A1* gene, one found in the three sisters and the other in an unrelated patient. However, there are other variants that could also be involved, to varying degrees, in the susceptibility to AFFs associated with BPs or in the underlying osteoporotic phenotype, so that our data would be compatible with a model in

Table 2a. Variants shared by the 3 sisters, found in the sequencing of the exome

Gen	Protein	Variant[a]	Effect on the protein	dbSNP[b]	ExAC[c]	Conservation[d]	SIFT[e]	PolyPhen[f]
GGPS1	Geranylgeranyl diphosphate synthase	chr1:g.235505746G>T	p.D188Y			700	**0.000**	**1.000**
LRRC1	Protein with leucine-rich repeats 1	chr6:g.53707020G>A	p.R91Q		4.946e-05	685	**0.050**	0.746
TUSC2	Tumor suppressor candidate 2	chr3:g.50363807T>C	p.H83R		8.244e-06	674	0.338	0.000
SYDE2	Rho GTPase activating protein	chr1:g.85634903G>T	p.L893I		8.339e-06	639	**0.018**	**0.997**
COG4	Subunit 4 of the conserved oligomeric Golgi complex	chr16:g.70553552C>T	p.G85D			627	0.150	0.735
EML1	Protein associated with microtubules	chr14:g.100360993G>A	p.R211H		6.611e-05	588	**0.030**	**0.963**
KDM4C	Specific lysine demethylase (K) 4C	chr9:g.6849579A>G	p.I170V	rs192832191 MAF=0,0004	2.471e-05	584	**0.000**	0.509
ERCC6L2	Repair protein by DNA cleavage	chr9:g.98718284A>T	p.I657L		8.278e-06	573	0.630	0.007
PGRMC1	Component of membrane 1 of the progesterone receptor	chrX:g.118377159C>A	p.P177H			573	0.130	0.742
FN1*	Fibronectin	chr2:g.216235149C>T	p.V2241I		8.245e-06	551	**0.009**	0.045
CYP1A1	Cytochrome P450 1A1	chr15:g.75015147G>A	p.R98W		0.000108	540	**0.000**	**0.998**
XAB2*	XPA 2 binding protein	chr19:g.7688142C>G	p.V385L		1.651e-05	535	**0.007**	0.600
GPR20	G 20 protein coupled receptor	chr8:g.142367729C>T	p.D99N	rs200892677 MAF=0.0004	3.324e-05	515	**0.000**	**0.998**
TMEM25	Transmembrane protein 25	chr11:g.118404174_ 118404176del	p.V239del			510	N/A	N/A
NGEF	Guanine nucleotide intercatalyst factor	chr2:g.233748153G>A	p.S542L		1.279e-05	500	0.350	**0.910**
NKAP	Activating protein of NFκB	chrX:g.119066123C>T	p.S265N	rs182030723 MAF=0.0006	6.847e-05	497	0.120	0.184
NVL	Nuclear protein containing valosin	chr1:g.224491450G>A	p.T312I		8.268e-06	474	**0.000**	**0.995**
FN1*	Fibronectin	chr2:g.216251538G>A	p.R1496W	rs139078629 MAF=0.003	0.004904	466	**0.005**	**0.998**
ATP6AP1	Subunit S1 of vacuolar proton ATPase	chrX:g.153664043G>A	p.V407I		4.561e-05	464	0.260	**0.990**
LURAP1L	Adapter protein rich in leucine 1	chr9:g.12821722G>A	p.R217H		4.948e-05	452	0.270	0.371
HEPHL1	Protein similar to hephaestin	chr11:g. 93839224G>A	p.W991*			451	**0.000**	N/A

Table 2a. (cont.)

Gen	Protein	Variant[a]	Effect on the protein	dbSNP[b]	ExAC[c]	Conservation[d]	SIFT[e]	PolyPhen[f]
NTPCR	Nucleoside triphosphatase related to cancer	chr1:g.233091444G>A	p.R59Q		5.779e-05	439	**0.034**	0.502
XAB2 *	XPA 2 binding protein	chr19:g.7688159G>C	p.T379R		1.652e-05	420	0.059	0.200
CHERP	Protein of the endoplasmic reticulum of calcium homeostasis	chr19:g.16631044C>T	p.R793H	rs202164310 MAF=0.0000	0.0001009	366	0.120	0.716
MEX3D	MEX3D RNA binding protein	chr19:g.1555839G>C	p.T560R	rs538022731 MAF=0.0002		366	**0.030**	N/A
BRAT1 *	ATM activator associated to BRACA1	chr7:g.2594007C>T	p.R20K	rs143390199 MAF=2e-05	1.651e-05	333	0.192	0.010
BRAT1 *	ATM activator associated to BRACA1	chr7:g.2580668G>A	p.T447M	rs368808380 MAF=0.0002	5.845e-05	333	0.110	0.275
CUL9	Culina 9	chr6:g.43154714C>T	p.T423I			251	**0.000**	**0.993**
ALPK1	α-kinase 1	chr4:g.113353195A>C	p.D831A		0.0001255	0	0.060	0.243
CD37	CD37 leukocyte antigen	chr19:g.49840212C>G	p.I63M		2.476e-05	0	**0.040**	0.028
IQCF6	F7 protein containing IQ motifs	chr3:g.51812782G>A	p.R61W			0	**0.010**	N/A
LFNG	Peptide O-fucosyl 3-β-N-acetylglucosaminyl transferase	chr7:g.2566829C>T	p.R375C		1.69e-05	0	**0.020**	0.772
MGA	Protein associated with the *MAX* gene	chr15:g.41988923C>T	p.S571L			0	0.130	N/A
POLI	Iota DNA polymerase	chr18:g.51820404T>C	p.V597A	rs543509008 MAF=0.0002	0.00024	0	0.590	N/A
SHC4	Protein 4 transformer SHC	chr15:g.49254675G>T	p.H180N			0	1.000	0.000
SMS	Spermine synthase	chrX:g.21958982G>C	p.G14R			0	0.350	0.002
SNAPC4	Polypeptide 4 of the snRNAs activating complex	chr9:g.139272279C>G	p.G1334R		2.675e-05	0	0.160	0.707

Table 2b. Other variants found in unrelated patients

Gen	Protein	Variant[a]	Effect on the protein	dbSNP[b]	ExAC[c]	Conservation[d]	SIFT[e]	Poly Phen[f]	Patient FAF
BRAT1	ATM activator associated to BRACA1	chr7:g.2580636C>T	p.E458L			333	0.568	0.000	AFU3
CYP1A1	Cytochrome P450 1A1	chr15:g.75014793T>A	p.S216C	rs146622566 MAF=0.0003	0.0001153	0	**0.004**	**0.987**	AFU1
MMP9	Matrix metallopeptidase 9	chr20:g.44641147T>C	p.M419T		8.242e-06	496	**0.000**	**1.000**	AFU3
MVD	Mevalonate diphosphate decarboxylase	chr16:g.88723957C>T	p.R97Q	rs376949804 MAF=3e-05	3.448e-05	0	0.448	0.009	AFU2
RUNX2	Transcription factor 2 related to Runt	chr6:g.45480010C>T	p.P296L	rs201584115 MAF=0.0004	0.0002066	642	**0.040**	**0.999**	AFU3

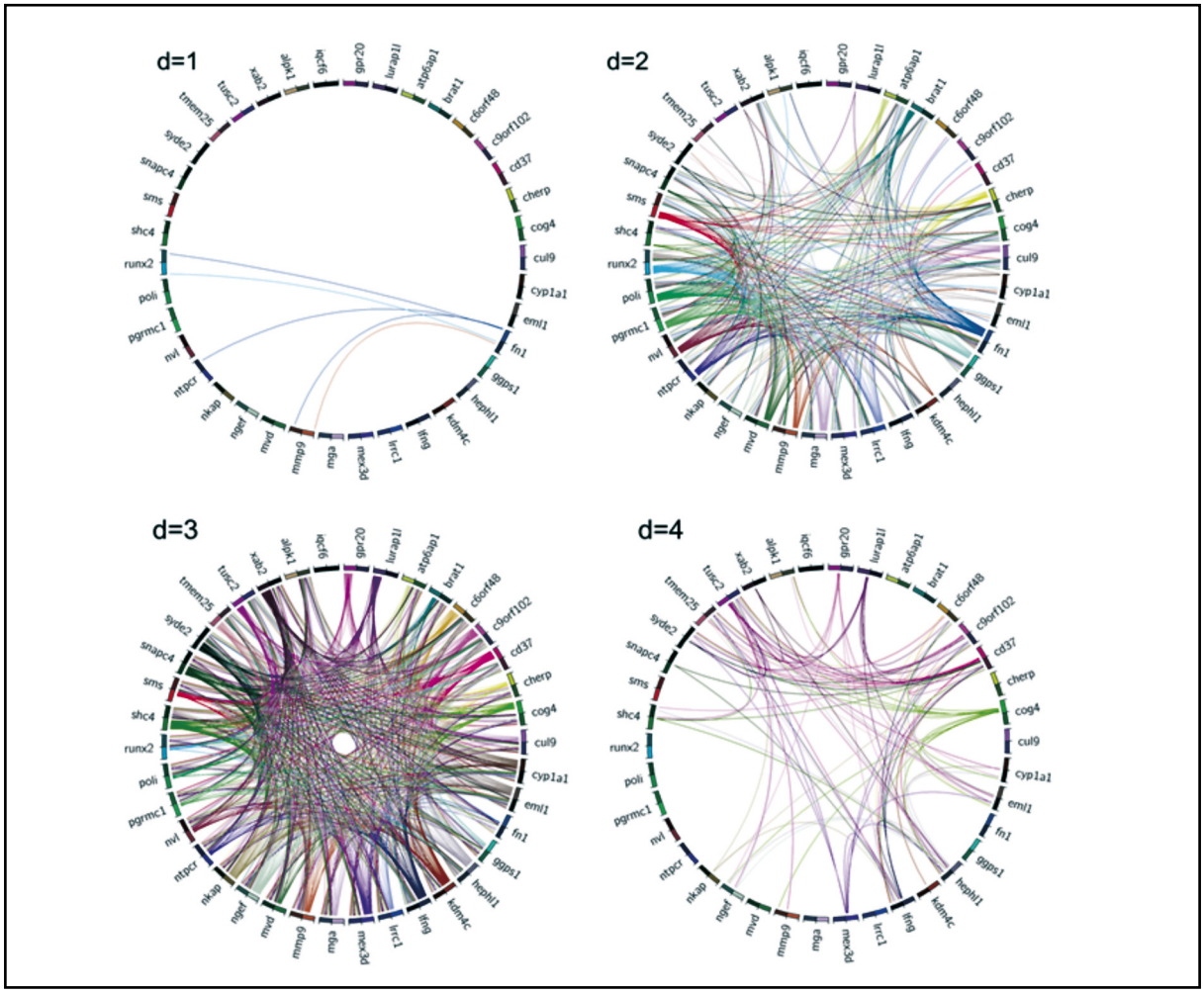
([a]): genomic position of the variant in the human reference genome GRCh37; ([b]): reference identifier number of the SNP (rs) and MAF (minority allele frequency) of the variants described; ([c]): allelic frequency of the variants described in the ExAC database; ([d]): PhastCons conservation score (0 to 1,000), with 1,000 being the most conserved locus and 0 a non-conserved locus; ([e]): SIFT: 0-0.05 harmful (in bold); 0.051-1 tolerable; ([f]): PolyPhen: benign 0-0.4; 0.41-0.89, possibly harmful; 0.9-1 pathogenic (in bold); (*): present in a mutant double allele.

which the accumulation of variants of susceptibility could contribute to the genetic base of the AFFs.

Epidemiological studies suggest that AFFs are related to prolonged treatment with BPs. Shane et al. described treatment periods of a median of 7 years[4]. The absolute risk of AFF associated with treatment with BPs is between 2 cases per 100,000 patients/year after 2 years of treatment and 78 cases per 100,000 patients/year after 8 years of treatment[25]. These data suggest that the duration of BP therapy would positively influence the risk of suffering these fractures. In our study, the cases of 6 patients with AFF after a long-term treatment with BPs are consistent with this association. In addition, the occurrence of AFFs in the 3 sisters suggests a genetic predisposition with a determining role in the pathology. This study has been the first exome analysis of AFF patients. We have prioritized rare, non-synonymous mutations, shared by the 3 sisters. No mutation was found in homozygosis or compound heterozygosis in any gene. These findings go against a pattern of recessive inheritance for these cases and are consistent with the fact that AFF is not a severe genetic disease that occurs during the early stages of life. However, in the dominant model, 34 mutated genes were found, some very important for bone metabolism. In an earlier work that aimed to discover the genetic causes of AFFs, an exome chip with >300,000 known coding variants was used and 21 overrepresented rare variants were found in 13 AFF patients[26]. However, none of these risk alleles was found in the patients analyzed in our study. Specifically, no variants were found in the *PPEF2* gene, the only one with a change significantly associated with the phenotype in the study by Pérez-Núñez *et al.*[26] This points to a heterogeneous genetic base for AFFs. In any case, it is important to point out that our methodological approach differs from that of the aforementioned study in that we analyzed the entire exome sequence, which allowed us to find variants not previously described.

In the present study, the only gene with mutations in the 3 sisters and in unrelated patients was *CYP1A1*. Recently, Peris *et al.*[27] sequenced this gene in 17 AFF patients and found another mutation in one of them. The *CYP1A1* gene encodes the cytochrome P450 1A1 enzyme that is involved in the metabolism of drugs and xenobiotics. It is a hydroxylase of aryl hydrocarbons and their poten-

Figure 1. Diagram of connectivity between gene pairs at distances 1 to 4. In the circles the symbols of the 37 AFF genes found in this study and their input and output connections are shown



tial exogenous substrates include polycyclic aromatic hydrocarbons, and is involved in the formation of different human cancers. Its endogenous substrates include eicosanoids, which can generate biologically active products that act in the vascular system, among others. This gene is also responsible for the hydroxylation of 17β-estradiol, estrone and vitamin D in extrahepatic tissues[28]. This is consistent with its role in bone biology, an idea supported by Napoli *et al.*[29], who demonstrated that the C4887A polymorphism was related to a significant increase in the catabolism of estrogen and a low femoral bone mineral density (BMD). postmenopausal women. Therefore, *CYP1A1* is presented as another potential susceptibility gene to AFFs, although the exact mechanism of its action on bone metabolism is still unknown and more studies are needed to elucidate it.

Among the other genes with variants in the three sisters, *FN1* encodes fibronectin, an extracellular matrix protein necessary for the regulation of the deposition of type I collagen by osteoblasts, essential for the mineralization of the extracellular matrix, and whose levels have been affected by treatment with BPs[30]. We found that the three sisters were carriers of a mutant double allele (p.V2241I and p.R1496W) in *FN1*, where the two mutations were considered as harmful by the predictors of pathogenicity. This altered fibronectin could affect bone mineralization and/or response to BPs and be related to the risk of AFF in these women. We also found mutated 2 regulators of small GTPases: *SYDE2* and *NGEF*. Their respective functions (activation of RHO GTPases and exchange of their guanine nucleotides) are clues about possible effects on osteoclastic function and response to BPs. The RHO GTPases are in the path of the mevalonate in a position below the site of action of the BPs, since they have to be prenylated (farnesylated or geranylgeranylated) for their correct cellular function. On the other hand, our gene/protein interaction network shows how *NGEF* is closely related to ephrins and ephrin receptors (Figure 2b), which have a key role in the mechanism of coupling between osteoclasts and osteoblasts[31]. Another group of genes mutated in the 3 sisters encode nuclear proteins with pleiotropic effects on gene expression and/or DNA repair (*KDM4C, XAB2, NVL, NKAP, ERCC6L2*). Of these, we highlight the *KDM4C* gene, which encodes a lysine-specific demethylase that contains a JmjC domain, which has been previously associated with the age of menarche[32], a biomarker for bone density.

Other genes found mutated in the sisters were the *PGRMC1* gene that encodes component 1 of the progesterone membrane receptor, and which was previously associated with premature ovarian failure[33]; the *COG4* gene (which codes for subunit 4 of the conserved oligomeric Golgi complex), which is relevant given the importance of transporting vesicles through the Golgi in osteoclasts[34]; and the *EML1* gene (which encodes a microtubule-associated protein) that may be important in relation to the primary cilium in osteocytes[35]. Overall, the functions and prior knowledge of 13 of the 34 genes mutated in the 3 sisters are consistent with their possible involvement in the pathology. These mutations were searched in the 3 unrelated AFF patients, with negative results.

However, through an approach of candidate genes, mutations in these patients were found in two key proteins for bone remodeling (RUNX2 and MMP9) and in another enzyme of the mevalonate pathway (MVD, mevalonate diphosphate carboxylase). RUNX2 is an essential transcription factor for osteoblastic differentiation[36], whereas MMP9 is a metalloprotease expressed in osteoclasts that degrades the extracellular bone matrix[37], affecting the architecture of trabecular bone and the structure of cortical bone[38]. For these reasons, both may be involved in the risk to the AFF. It is known that RUNX2 activates gene expression of MMP9[39] and this interaction may have synergistic effects on the biomechanical properties of bone in patient AFU3, which has both mutations (Note: this interaction is not shown in Figure 2a so that other interactions can be shown clearly). Finally, in the AFU2 patient, a change mutation was found in the *MVD* gene, adding a second mutated protein from the mevalonate pathway. Figure 3 shows, in the context of bone cells, the proteins encoded by the genes we have found mutated and whose function in bone is known or predicted.

Taken together, all these rare variants can be part of a genetic background associated with developing bone changes that give rise to AFFs and the possible negative interaction with BPs. It is likely that several genes with small additive effects, and their interactions, are involved in AFFs related to BPs. In addition, each individual patient could be a carrier of different specific genetic variants.

The strengths of this study are the possibility of analyzing 3 sisters with AFF and the sequencing approach of the complete exome, which lacks a previous hypothesis. In this sense, we were able to identify harmful mutations in genes belonging to the mevalonate pathway, as well as other genes related to bone metabolism. On the other hand, the low number of patients and controls studied is a limitation of the study. Further studies of exome sequencing of additional AFF patients and of non-fractured patients with a long-term treatment with BPs (acting as controls) will be necessary to clarify the precise role of these genes and mutations. Despite the biological plausibility of the damaging effect of the mutations found, the replication of these findings is needed.

The identification of the genetic background for atypical fractures of the femur opens the door to the future development of tools for diagnosis and prediction of the risk of suffering this type of fracture to determine the suitability of BP treatment.

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

Figure 2. Details of the interaction network between genes/proteins. The color of the interior of the nodes indicates subexpression (yellow), overexpression (blue) or no change of expression (white) in osteoclasts treated with alendronate or risendronate (data from Yuen et al., 2014[24]). The external color identifies the genes as drivers (mutated in our patients) in lilac, upstream of the genes mutated in green, and others in gray. a) Interactions of the *GGPS1* and *CYP1A1* genes at distance 2 (and some of the *MMP9* gene at distance 1). Note: some connections have been omitted for the clarity of the figure. In particular, nodes RUNX2 and FN1 have not been expanded to show all their connectors. b) Interactions of the *SYDE2* and *NGEF* genes at a distance 1

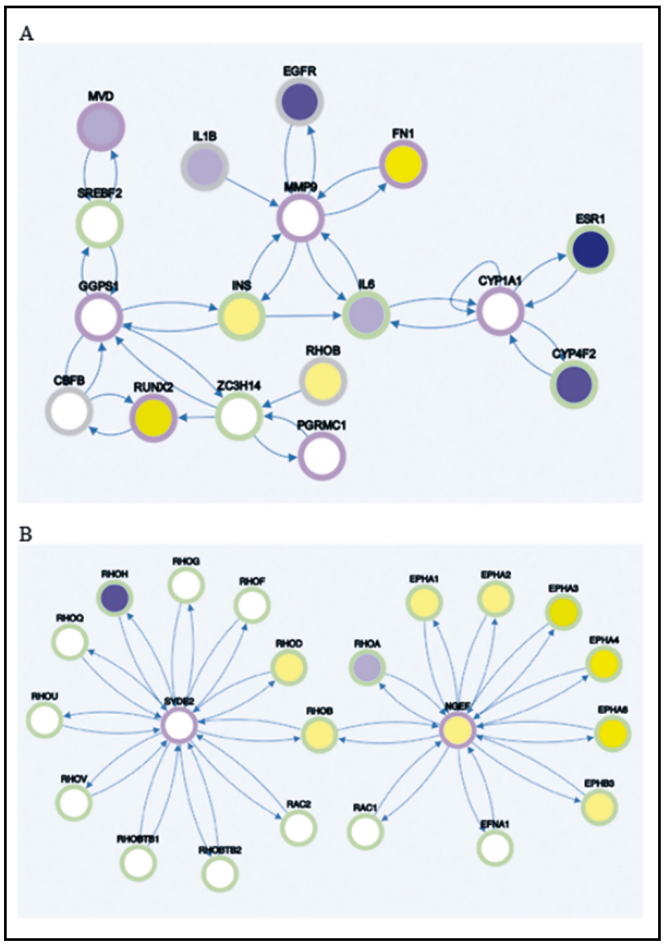


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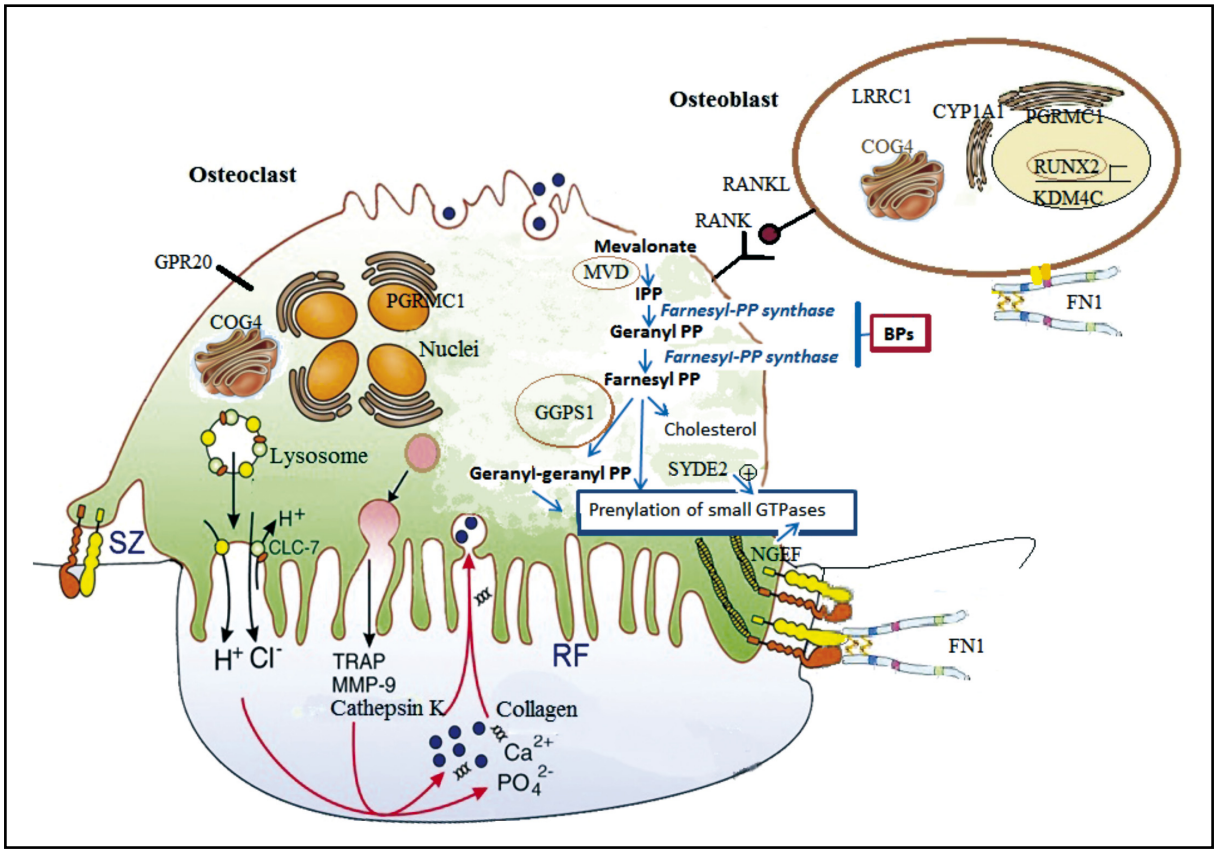
Figure 3. Proteins encoded by the mutated genes in AFF patients in this study and related to bone function



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Functional studies of DKK1 variants present in the general population

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Summary

Objective: In recent decades, genes associated with bone mass and osteoporotic fracture risk have been identified, several of which belong to the Wnt pathway. In this project, the functionality of 7 missense mutations of the gene *DKK1* –an inhibitor of the Wnt pathway– present in the general population was studied.

Material and methods: In vitro studies of the luciferase reporter gene were carried out to measure Wnt pathway activity in the presence or absence of wild-type or mutated DKK1, and western blot studies, to evaluate if the different mutations affect its synthesis and/or stability.

Results: The DKK1 protein with the p.Ala41Thr variant shows lower pathway inhibitory activity compared to the wild-type protein. Significant differences were also observed between the experiments performed in the absence of DKK1 and those that include DKK1 with the p.Ala41Thr mutation. Western blots showed that the amount of protein was similar for all variants, both mutated and "wild-type, so the loss of p.Ala41Thr activity did not seem to be due to a lack of protein. The rest of the mutations did not show different behavior from that of the wild DKK1 protein.

Conclusions: The missense variant p.Ala41Thr of the DKK1 protein, with a population frequency of 0.013%, shows a partial loss of its inhibitory function, which is not due to the lack of expression. This gene variant could lead to an increase in bone mineral density in those people in the general population who carry this mutation.

Key words: *DKK1, functional studies, missense variants, luciferase, Wnt pathway, High Bone Mass (HBM), osteoporosis.*

Introduction

The Wnt pathway's role in regulating bone remodeling has been demonstrated in multiple studies. On the one hand, polymorphisms have been described in several genes of the Wnt pathway that show an association with bone mineral density (BMD) and the risk of fracture[1-6]. Rare or infrequent mutations have also been described in various genes of the Wnt pathway, which cause more rare bone phenotypes, such as osteoporosis-pseudoglioma (OPPG, OMIM 259770)[7], autosomal recessive osteogenesis imperfecta of type XV (OMIM 615220)[8], and osteosclerosis (OMIM 144750)[9]. The Wnt pathway begins with the formation of a heterotrimeric complex between the Frizzled receptor, the LRP5 co-receptor and the Wnt ligand. Once this complex is formed, β-catenin accumulates in the cytoplasm and translocates to the nucleus where it can activate the transcription of numerous target genes. In osteoblasts, the Wnt pathway has been shown to activate the transcription of genes that clearly contribute to bone formation[10]. In addition, this pathway is finely regulated by a series of extracellular inhibitors, including the protein sclerostin, encoded by the *SOST* gene, and the DKK1 protein, encoded by a gene with the same name. These two proteins perform their function, preventing the formation of the heterotrimeric complex. The proteins sclerostin and DKK1 thus form other heterotrimeric complexes, together with LRP5 and LRP4 (in the case of sclerostin)[11,12] and together with LRP5 and Kremen (in the case of DKK1)[13].

The transgenic over-expression of the *DKK1* gene in osteoblasts produces a relative decrease in the number of osteoblasts compared to that of osteoclasts, thus producing a decrease in bone formation. Similarly, in mice, the homozygous deletion of the *DKK1* gene is lethal, but deletion in heterozygosis presents a phenotype of bone overgrowth (high bone mass)[14,15].

In the past decade, thanks to the direct effect on osteoblastogenesis inhibition and the indirect activation of osteoclastogenesis[16], sclerostin and DKK1 have become interesting targets in osteoporosis treatment. Regarding DKK1, antibodies have been developed (BHQ880, DKN-01 and PF-04840082), the first of which in the clinical trial phase in postmenopausal women with low bone mineral density (BMD)[17-19].

In our group's previous study[20], *DKK1* was sequenced to identify variants that could explain the high bone mass (HBM) phenotype , defined by a femoral + lumbar Z-score > 4, present in 15 women. In one of them, a missense mutation was found (p.Tyr74Phe) that co-segregated with the HBM phenotype in the family. In another study of *DKK1* gene sequencing in postmenopausal women of the BARCOS cohort, we found another missense mutation (p.Arg120Leu) in another woman with HBM[21]. In addition to these mutations, in the general population there are other variants of change of direction in DKK1 (http://exac.broadinstitute.org/), whose effect in terms of bone mass has not been reported.

In the present work, we have conducted *in vitro* studies of the mutations p.Arg120Leu and p.Tyr74Phe, together with other missense mutations of DKK1 frequent in the general population (p.Met16Leu, p.Ala41Thr, p.Pro84Leu, p. Ala106Thr, p.Ser157Ile), to assess their possible involvement in bone phenotypes.

Material and method

Expression and mutagenesis vectors

The Wnt1-V5 mouse expression vectors, mesdc2, human wild-type LRP5, pRL-TK, PGL3-OT and DKK1-FLAG22, were courtesy of Dr. Wim van Hul (Antwerp, Belgium). Mutations p.Met16Leu, p.Ala41Tyr, p.Tyr74Phe, p.Pro84Leu, p.Ala106Thr, p.Arg120Leu, p.Ser157Ile were introduced into the DKK1-FLAG expression vector using the Quick Change Site-Directed Mutagenesis kit (Stratagene). The presence of mutations and the absence of errors were verified by Sanger sequencing.

Cell culture, production of conditioned medium and western blot

HEK293 cells, cultured with DMEM medium supplemented with FBS (10% V/V, Gibco, LifeTechnologies) and 1% streptomycin-penicillin (Gibco, LifeTechnologies) and maintained in incubators at 37°C at 5% in CO_2. Were used. 3 x 10^5 cells were seeded per well in 6-well plates, 24 h before transfection. 2,000 ng/well of the mutated or wild-type DKK1-FLAG plasmids were transfected. The transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 24 h, the medium was changed, reducing from 2 to 1 ml of DMEM, without FBS (Fetal Bovine Serum) or antibiotics. 48 hours after transfection, the supernatant of each condition was collected. The proteins of the conditioned medium were concentrated using Amicon Ultra filters (Millipore) and quantified by the BCA assay (Pierce). The proteins from the conditioned media (4.5 µg/lane) were separated by electrophoresis in a polyacrylamide gel with SDS (SDS-PAGE) and transferred to a nitrocellulose membrane. For the western blot analyses, Abcam ab109416 antibodies against DKK1 and ab2413 were used against the extracellular protein fibronectin, as a load control. The images were developed using a secondary antibody conjugated with peroxidase (Sigma-Aldrich). For each mutant conditioned medium was obtained in 2 different days and the analysis was carried out by western blot 2 times with these conditioned media.

Gene reporter assays

HEK293 cells were used, cultured as indicated in the previous section. 10^4 cells were seeded per well in 96-well plates, 24 h before transfection. Up to 5 plasmids were cotransfected in HEK293 cells: Mouse Wnt1-V5 (3.2 ng), mesdc2 (6.4 ng), human wild-type LRP5 (6.4 ng), pRL-TK (8 ng), and pGL3-OT (160 ng). In addition, depending on the experiment, the wild or mutated plasmid DKK1-FLAG (0.6 ng) was also co-transfected. If necessary, the

empty vector pcDNA3 was used to equal the total amount of DNA from each experiment. The transfection was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. 48 h after transfection, the cells were lysed and the luciferase activity of Photinus pyrali and Renilla reniformis was measured using a Glomax Multi+luminometer (Promega) following the instructions of the Dual-luciferase reporter assay (Promega). Each experiment included 5 replicates and was repeated independently in 3 separate experiments.

Statistical analysis

A one-way blocked ANOVA model was carried out for each mutant taking into account the test factor, the day as a blocking factor and the response variable the relationship between the activities of the luciferases (Photinus pyrali vs. Renilla reniformis). Blocking is a technique to deal with the nuisance factor and this can influence response. For each mutant protein, the test factor has the following levels: control (refers to the activity of the luciferase resulting from the endogenous Wnt pathway), the activator (luciferase activity produced by the Wnt pathway in the presence of Wnt and exogenous LRP5), the inhibitor (activity in the presence of the wild-type DKK1 inhibitor) and mutant (each of the mutant DKK1 proteins). The TukeyHSD test was used to carried out the post hoc test for multiple group comparisons. The ANOVA tests were done using the program R studio v.3.4.0, and values of $p<0.05$ were considered significant. All data were evaluated for normality, homogeneity of variance and detection of outliers.

Results

Expression, secretion and stability of mutated proteins

A western blot assay was performed to check if the DKK1 mutant proteins are correctly located in the extracellular space, using a culture of HEK293 cells, which express high amounts of wild-type DKK1 or mutated DKK1. The results show that, in all cases, the different mutated DKK1 proteins were detected in the extracellular space (Figure 1) and at levels equal to or higher than those of the wild-type protein.

Activity of mutated DKK1 proteins

To test the inhibitory activity of mutant DKK1 proteins on the Wnt pathway, we performed a reporter gene assay (luciferase), specific for this pathway (Figure 2).

The results of the endogenous condition in which the plasmids pRLTK and pGL3-OT have been co-transfected are shown in Figures 2A and 3 which represent the Wnt pathway activity in HEK293 cells.

In the active condition, in addition to pRLTK and pGL3-OT, the vectors expressing Wnt1 and LRP5 have been co-transfected. Wnt1 acts as a ligand activator of the pathway and LRP5 as a co-receptor, two essential elements for the pathway activation. In this condition (Figure 2B) the activity of luciferase has been increased 3 times, on average, compared to the endogenous pathway (Figure 3, activator).

The inhibited pathway contained the same vectors as the active condition but in addition the vector expressing the protein DKK1-WT (wild-type protein) was co-transfected. In this condition, the Wnt pathway has been inhibited, by sequestering the LRP5 co-receptor (Figure 2C). In these experiments, luciferase activity has been increased 2.2-fold over the endogenous condition and has been significantly lower than that of the activated pathway (Figure 3, inhibitor).

When the functionality of the mutants of DKK1 has been verified, the different vectors of the inhibited pathway have been co-transfected, but substituting that of DKK1-WT for those expressing the different mutated DKK1. For the mutant proteins DKK1-p.Met16Leu, DKK1-p.Tyr74Phe, DKK1-p.Pro84Leu, DKK1-p.Ala106Thr, DKK1-p.Arg120Leu and DKK1-p.Ser157Ile no significant differences were found in the inhibitory activity compared with the DKK1 WT protein (data not shown).

In contrast, in the presence of the mutant protein DKK1-p.Ala41Thr, luciferase activity has been observed which is significantly greater than that of the pathway inhibited by DKK1-WT (Figure 3), and in turn significantly lower than that of the active route.

Discussion

The *DKK1* gene encodes a protein of the same name, which acts in the extracellular space as an inhibitor of the Wnt signaling pathway. Numerous studies have associated the Wnt pathway with bone formation, while blocking it with sclerostin or DKK1 has been associated with greater bone loss and risk of fracture. The search for gene variants that may explain Wnt pathway regulation in the general population may open a very relevant field of research in osteoporosis study. In this work, we have studied the inhibitory function of 7 mutant DKK1 proteins on the Wnt pathway and we have observed that the mutant protein DKK1-p.Ala41Thr shows a partial loss of its inhibitory function, which is not due to the loss of its expression. The activity of the DKK1-p.A41T protein is reduced by approximately 50% compared to the protein DKK1-WT. The mutation, at amino acid 41, is not found in the LRP5 binding domain (amino acids 189-263), but it does affect the NAIKN motif (amino acids 40-44), which is crucial for binding to LRP5 and LRP6 proteins and it is conserved in all inhibitors of the Wnt pathway[23].

According to the ExAC database, the population frequency of the variant p.Ala41Thr is 15 heterozygotes in 60,000 adult individuals free of serious diseases. Given our result of loss of inhibitory activity of this variant of DKK1, we could infer that the associated phenotype would be of a higher non-pathogenic bone density. From this frequency, we estimate that in Spain there are about 6,000 carriers of this variant in heterozygo-

sis. On the other hand, there is a single reference to the mutation p.Ala41Thr that associates it with pathology, specifically the Chiari type I malformation (CMI)[24]. This disease is characterized by a defect in the development of the occipital bone and the posterior fossa (PF) and the consequent hernia of the cerebellar amygdala. It will be interesting to study the possible relationship between mutations in *DKK1* and this disease, which, in many cases, is asymptomatic and undiagnosed.

No differences were found in the activity of the remaining mutant proteins and the DKK1 WT protein. These results coincide with the results found by Korvala et al.[25] for the mutation p.Arg120Leu. These authors found this mutation in a patient with primary osteoporosis, a phenotype diametrically opposed to the phenotype of the HBM woman where we found the mutation. This same mutation is found in patients with Paget's disease (PDB)[26] and its frequency in patients is twice that of controls, although the difference is not significant.

None of the seven mutations tested is in the domain that affects the Wnt signaling pathway (LRP5 binding domain: amino acids 189-263), and only p.Ala41Thr affects the NAIKN motif. This

Figure 1. Expression levels of the wild-type or mutated DKK1 protein analyzed by western blot. HEK293 cells were transfected with expression vectors of the different DKK1 variants indicated in each lane. The resulting conditioned media, properly concentrated, was used for this analysis. In each lane 4.5 ug of total protein was loaded. The extracellular protein fibronectin has been used as load control

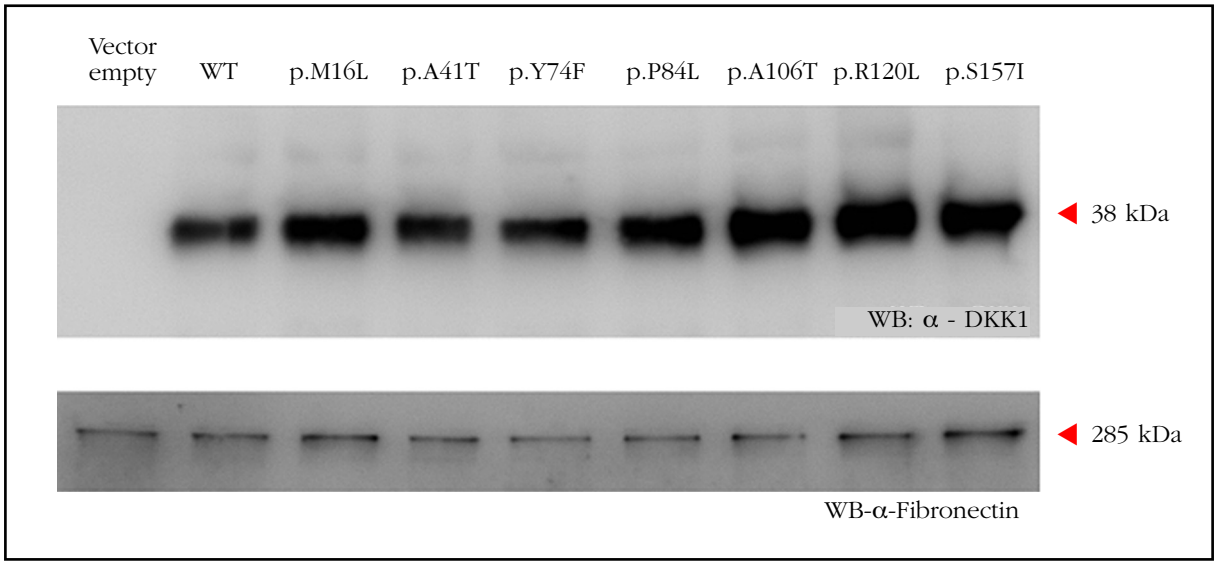
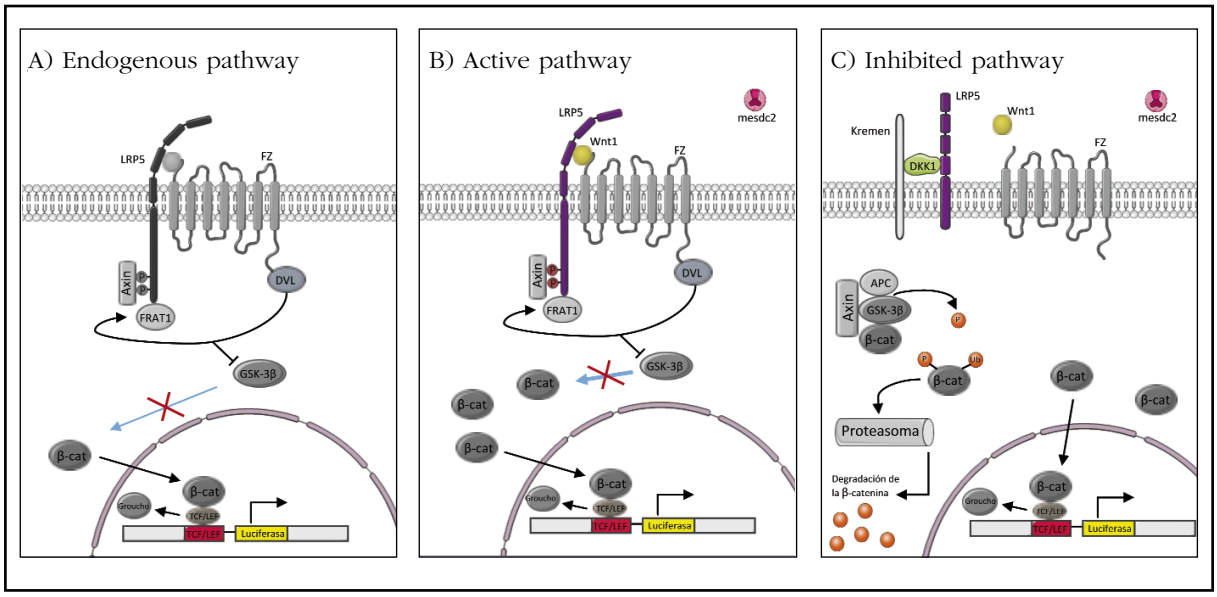


Figure 2. Reporter gene assay design. A) Endogenous condition: we transfected the pRLTK and pGL3OT plasmids. B) Active condition: we transfected plasmids pRLTK, pGL3OT, Wnt1, LRP5 and mesdc2. C) Inhibited condition: we transfected plasmids pRLTK, pGL3OT, Wnt1, LRP5, mesdc2 and DKK1-WT. In gray the endogenous elements of the HEK293 cells of the Wnt pathway, in color the transfected elements in each condition



could be a reason why no differences in inhibitory activity have been observed in 6 of the 7 mutated DKK1. Alternatively, these DKK1 mutants would show differences in inhibitory activity lower than those that can be detected with the sensitivity of the reporter gene assay that has been used. A limitation of the study would be that the assay carried out involves the co-transfection of several vectors to have high values of luciferase activity, which gives it a high variability. Another limitation would be that the expected effect of these mutations is small, since they are variants present in the general population. This question can only be solved when there is a trial with a higher sensitivity.

In conclusion, in our study, DKK1 protein (p.Ala41Thr) shows a partial loss of its inhibitory function, which is not due to its lack of expression. This could lead to an increase in bone mineral density in people of the general population who carry this mutation.

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

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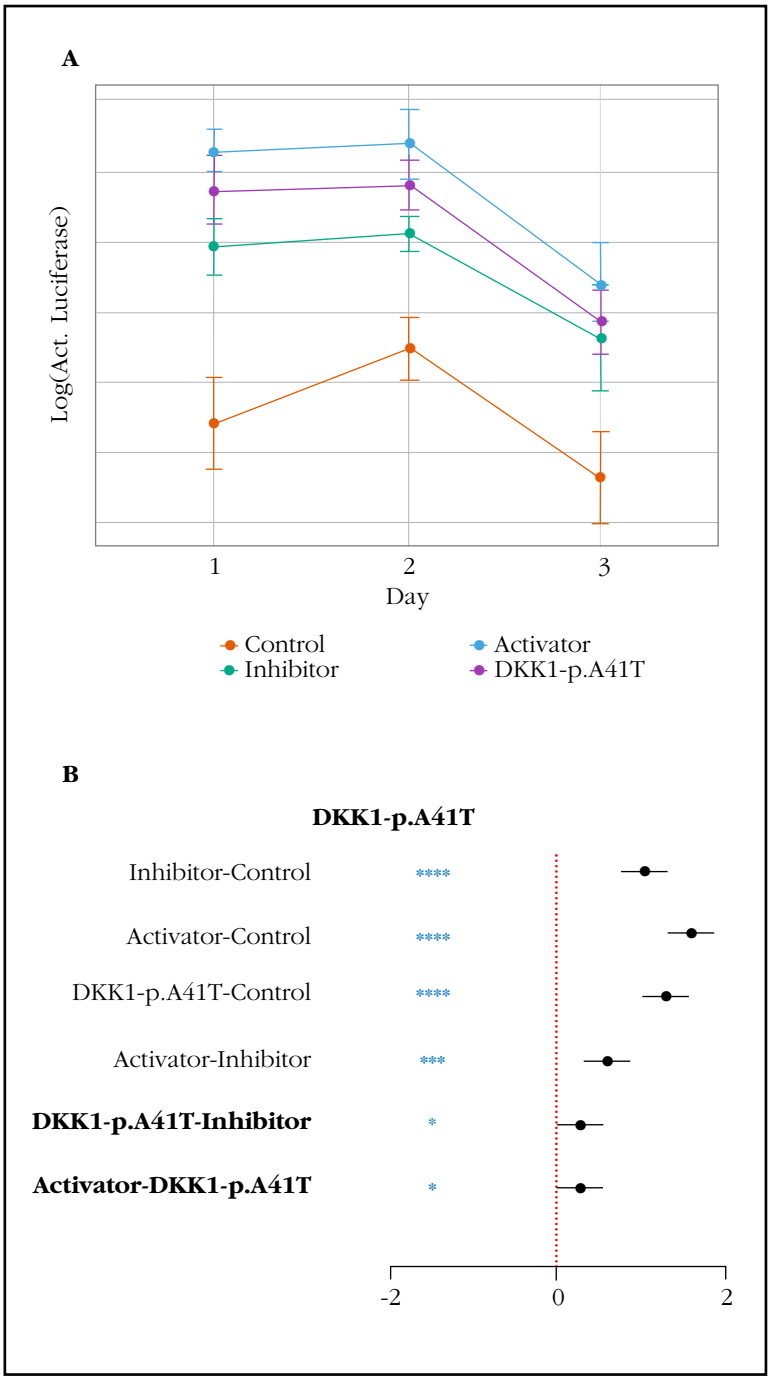
Figure 3. Activity assay of the DKK1-p.Ala41Thr protein. A) Graph of interactions following the model, Yij = μ + day + test + day: test + ϵijk. The logarithm of the mean ratio of luciferase activities (Photinus pyrali/Renilla reniformis) with its confidence interval is shown on the Y axis. in the X axis the three days in which the experiment has been tested are shown. B) Tukey test of multiple comparisons between the different conditions tested. Significance levels are shown by means of the code: (****)<0.000001, (***)<0.0001, (**)<0.01, (*)<0.05



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Bone tissue mechanical strength is independent of age in healthy individuals

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Summary

Objective: Impact microindentation (IMI) is a technique that allows the measurement of mechanicalbone tissue resistance *in vivo*. IMI has proven to provide useful information on the evaluation of skeletal diseases, but the effect of age on the bone property that is measured by this technique is unknown. This study aims to analyzethe relationship between age and MIH.

Material and methods: Bone Material Strength index (BMSi), IMI's output variable, was measured in 69 healthy women (median age: 49 years, range: 30-81 years) and 19 healthy men (median age: 34 years, range: 24-98 years). The correlation between BMSi and age was analyzed by linear regression. The association between BMSi and age was evaluated by ANOVA after adjusting for body mass index. The potential effect of postmenopausal estrogenic depletion on BMSi was studied by comparing the younger vs the older subset of women through a t-student test.

Results: Linear regression analysis showed that BMSi was not correlated with age in either men (R^2=0.0016, p=0.74) or women (R^2=0.076, p=0.25). Similarly, the BMI-adjusted ANOVA model revealed a lack of association of BMSi with age in men (p=0.78) and women (p=0.73). Finally, there were not significant differences on BMSi detected between the younger and the older subset of women (p=0.8).

Conclusions: Bone tissue mechanical resistance in healthy individuals is independent of age and postmenopausal estrogenic depletion.

Key words: *impact microindentation, Bone Material Strength index (BMSi).*

Introduction

Osteoporotic fractures pose a serious public health problem given their high prevalence and enormous impact in terms of morbidity, mortality and economic cost[1]. Hence there is considerable interest in understanding the underlying pathophysiology of bone fragility, which, from a mechanical standpoint, is determined by bone strength. Bone resistance, in turn, comes from the integration of bone mineral quantity, bone architecture, and the material properties of bone.

The mineral quantity of the bone is usually measured by bone densitometry (DXA), the most commonly used, standardized method for assessing bone mass and fracture risk[2]. Bone architecture, both at the micro- and macroscopic level, is examined using different imaging techniques, including high-resolution peripheral quantitative tomography, bone magnetic resonance and the more accessible Trabecular Bone Score[3]. However, the material properties of bone are difficult to assess due to its high complexity, reflected in its multiple constituents including non-collagenous proteins, crystallinity, hydration of bone tissue, and the characteristics of mineralization and collagen, among others[4,5]. Furthermore, as researchers need bone tissue samples for analysis, the study of these properties has traditionally been restricted to a few centers specialized in bio-mechanics.

Microindentation has been developed as a technique to measure the material properties of bone easily and non-invasively. However, the property specifically measured has not yet been determined, so for the time being, the mechanical strength of the bone is evaluated globally. This technique involves measuring the penetration distance of a needle in the cortical bone to gauge its mechanical resistance[6]. The procedure is usually carried out on the anteromedial side of the tibia in a practical, safe and painless way[7]. There are currently two types of clinical microindentation: the cyclic microindentation, using the BioDent® instrument (Active Life Scientific Inc., Santa Barbara, USA). The other is impact microindentation (IMI), carried out with the OsteoProbe®. Several clinical studies can provide relevant information on bone strength and risk of fracture with both types[6,8]. However, given its greater manageability, OsteoProbe® has replaced BioDent® in clinical studies. Despite its increasing use, there are still many unresolved basic issues surrounding IMI implementation including the effect of age on bony material properties. We examined the influence of age on bone tissue mechanical strength in a cohort of healthy men and women.

Material and methods

Participants

Healthy volunteers older than 18 years of age were recruited consecutively from Internal Medicine outpatient lists without bone metabolism-related diseases.

Those individuals with the following criteria were excluded from the study:

- History of fragility fractures or traumatic fractures of the tibia.
- History of primary bone diseases (including osteoporosis), secondary bone diseases, deformities in the lower extremities of congenital or acquired origin, and bone metastasis.
- History of diabetes mellitus, chronic kidney disease and severe liver failure.
- Previous or concurrent treatment with glucocorticoids, aromatase inhibitors, androgen deprivation therapy, chemotherapy and antiresorptive agents or osteo formers (bisphosphonates, teriparatide, denosumab, strontium ranelate and selective modulators of the estrogen receptor).

Participants' height and weight were measured to calculate the body mass index (BMI, kg/m^2).

The study protocol was approved by the Ethics Committee of the Mar Health Park and written informed consent of all the participants obtained.

Impact Microindentation

Impact microindentation (IMI) was evaluated using OsteoProbe®, a hand-held device with an impact mechanism, a disposable probe with a conical tip (radius of tip sharpness: <10 µm) and a displacement transducer. The procedure has been described in detail previously[7]. Prior to microindentation, a local anesthetic (2% mepivacaine) is applied to the anteromedial part of the non-dominant tibia. The probe is then inserted perpendicular to the bony cortex in the anesthetized region until it reaches the bone surface. The device is slowly compressed until it reaches a pre-load resistance of 10 Newtons (N), after which an impact load of 30 N is automatically activated. The displacement transducer measures indentation depth. The operator can eliminate the measurements that are considered incorrect.

After 8 valid indentations separated by approximately 2 mm, 5 additional indentations are made with the same probe in a polymethyl methacrylate (PMMA) block for calibration. The value obtained in the IMI is the Bone Mineral Resistance Index (or BMSi, from Bone Material Strength index), which is defined as 100 times the relation between the harmonic mean of the distance of the 8 bony indentations and that of the 5 indentations in the PMMA block. Nine different operators with experience in the technique carried out the measurements in our study.

Statistical analysis

Separate analyzes were carried out for women and men. Descriptive values are shown using mean and standard deviation, as well as median and total range, as appropriate. The correlation between age and BMSi was represented by a linear regression, and its association with BMI-adjusted ANOVA evaluated. Due to the lack of clinical information on the menstrual status of the participants, the potential effect of estrogen deprivation on the mechanical resistance of bone tissue was analyzed by comparing the BMSi of women between 20-39 years (most likely premenopausal) with women >60 years (most likely postmenopausal) using Student's t test.

The study figures were obtained through the Prism 7 program (GraphPad Software, La Jolla, California, USA). The statistical analyzes were performed with the SPSS program version 23 (IBM Corp®, Armonk, New York, USA), accepting as significant the results with $p<0.05$.

Results

For our study, 69 women and 19 men of Caucasian origin were recruited. The participants' characteristics and the BMSi measurements are shown in table 1. The coefficient of inter-operator variation was less than 5%.

Linear regression analyzes showed that BMSI does not correlate with age in women (R^2=0.076, p=0.25) nor in men (R^2=0.0016, p=0.74) (Figure 1). Likewise, no significant associations were detected between the BMSi and the age in the ANOVA analysis adjusted for BMI neither in women (p=0.73) nor in men (p=0.78). Finally, no significant differences were observed in the BMSi between the subgroup of women aged 20-39 years and those older than 60 years (p=0.8) (Figure 2).

Discussion

In the present study, the influence of age on the mechanical resistance of bone tissue measured by IMI in a cohort of healthy men and women was evaluated. The results indicate that bone tissue resistance is not determined by age in women or men, and that therefore, it is not affected by aging. Furthermore, no BMSi differences were found between the subset of younger women versus the subset of older women which would indicate that the depletion of estrogen that accompanies menopause does not exert a significant effect on the mechanical resistance of the bone tissue.

Bone microindentation has emerged as a promising new tool to evaluate bone mechanical resistance in living individuals[6-8]. Although it is still unclear which physical properties are specifically measured, several clinical studies reveal that this technique has a good discriminant capacity between patients with and without fragility fractures[9-11], although studies in geriatric populations with osteoporotic fractures show discrepancies[12].

The measurements that result in an altered BMSi seem to be especially informative in those conditions associated with an increased fracture risk that are not explained by abnormal BMD values[13-16].

Given the increasing use of IMI as a complementary technique for assessing bone health in clinical research and its potential future role in clinical practice, it is imperative to clarify the possible effects of physiological factors, such as age, on the mechanical resistance of the bone tissue.

Our study results indicate that BMSi is not significantly affected by aging or estrogenic depletion. Mirzaali MJ, et al. observed through micromechanical studies in cadaver bone that the properties of microindentation in the elderly were constant with age[17] which concurs with our study. On the contrary, aging and estrogenic depletion reportedly exert a negative effect on BMD and bone architecture[18,19]. This reinforces the notion that the microindentation technique measures a very specific characteristic of bone strength or bone quality completely different from other techniques available to date. Microindentation may cause the separation of the protein-based "glue" proteins, which hold together the mineralized collagen fibrils, a property that would constitute the first barrier of resistance to fracture[20].

Currently, bone tissue resistance is considered to be genetically determined, but, at the same time, clinical studies show that it can be negatively influenced by non-genetic factors, such as a deficient glycemic control[13,15,21], an excess of tissue local adipose[16], treatment with glucocorticoids[22], excess growth hormone[23], chronic kidney disease[24,25] and HIV infection[26]. In addition, alterations in certain signaling pathways and in the intracellular storage of lipids also seem to affect the mechanical resistance of bone tissue as has been observed in studies on the diseases of Camuratti-Englemann[27] and Gaucher type 1[28], respectively.

Our study has several limitations. First, the small number of subjects included in the study limits the generalization of our findings. Another limitation is that the factors that can affect the mechanical resistance of the bone tissue are not yet fully known. Therefore, these factors have not been introduced as co-variates in the statistical model for the adjustment of the confounding factor. This has been tried to compensate by means of the strict exclusion criteria used at the moment of the recruitment of the individuals and thus control the possible heterogeneity of the studied cohort.

Table 1. Characteristics of the participants

	Women (n=69)	**Men (n=19)**
Age, years (median, range)	49, 30-81	34, 24-98
BMI, kg/m² (mean ± SD)	24.3±4.5	24.6±3.1
BMSi (mean ± SD)	82±7.4	88±7.6

BMI: body mass index; DE: standard deviation; BMSi: Bone Material Strength index.

Figure 1. Linear regression analysis performed to evaluate the correlation between the Bone Material Strength index (BMSi) and age in healthy men and women

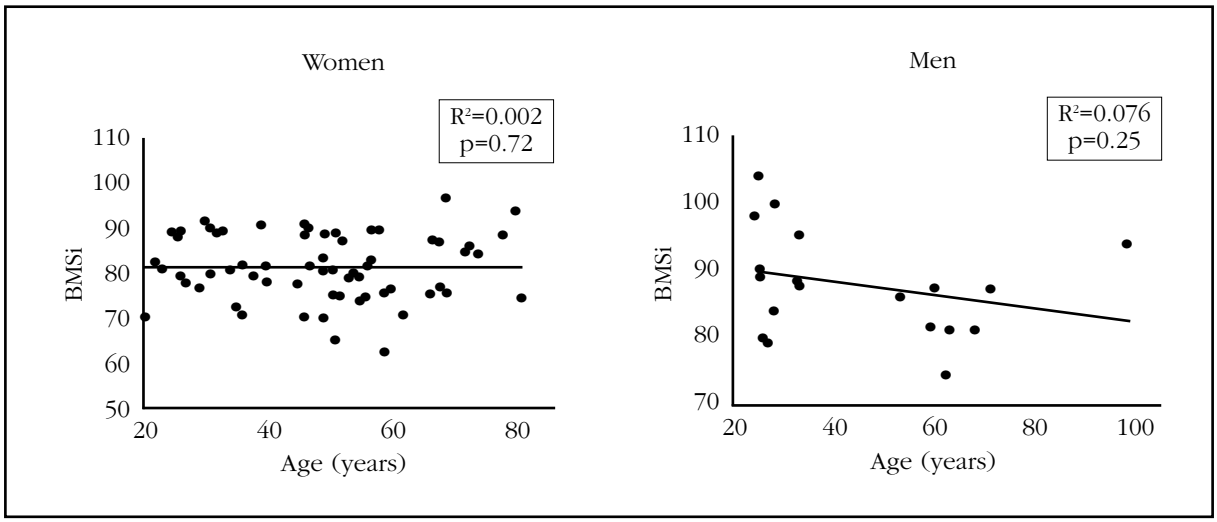
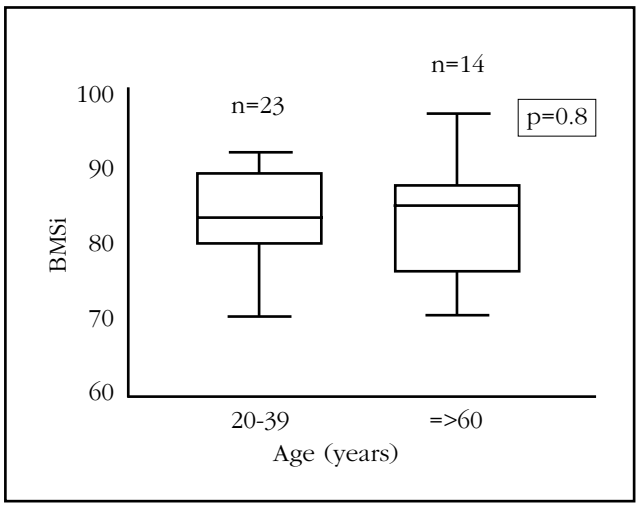


Figure 2. T-test carried out to compare the BMSi between women aged 20-39 years and women >60 years of age as an indirect measure of pre and postmenopausal status, respectively



Another limitation lies in the fact that this technique is performed exclusively on the cortical bone of the anteromedial tibia, so the generalization of BMSi results to other skeletal sites is debatable. However, we believe that the values obtained by microindentation in the tibia reflect the mechanical strength of the bone globally, since clinical studies have shown an inverse correlation between BMSI values and the incidence of osteoporotic fractures in other skeletal locations such as hip, and even in bones with a greater trabecular component, such as the vertebrae[10,11]. Finally, the data on the menstrual status were not collected, thus limiting the evaluation of the effects of menopause on the mechanical resistance of the bone tissue. This problem was counteracted by categorizing the subgroup of younger women as premenopausal and the subset of older women as postmenopausal.

In conclusion, the mechanical resistance of the bone tissue does not seem to be affected by aging and estrogen-related depletion related to menopause. Additional studies are needed to corroborate these findings in order to facilitate the implementation of the IMI in research and clinical practice.

Conflict of interests: Adolfo Díez-Pérez declares that he owns shares of Active Life Scientific, the manufacturer of microindentation devices. The remaining authors declare that they have no conflicts of interest.

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Factors secreted by bone cells induce intracellular calcium accumulation and cyclic AMP and activation of ERK 1/2 in prostate cancer cells; evaluation by fluorescence techniques in living cells

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Summary

Objectives: To analyze in prostate tumor cells the effects caused by the secretome of bone cells on proliferation and on intracellular signaling pathways related to the progression of prostate cancer.

Materials and methods: The effects of secreted factors present in conditioned media of pre-osteoblasts MC3T3-E1 and osteocytes MLO-Y4 on the proliferation of metastatic prostate adenocarcinoma cells PC-3 were characterized using trypan blue staining. The effects of media conditioned by MC3T3-E1 and MLO-Y4 cells on intracellular signaling molecules involved in the tumor progression of prostate adenocarcinoma cells PC-3 were observed by fluorescence techniques in living cells. The accumulation of intracellular calcium was studied using the fluorescent calcium indicator Fluo-4AM and the generation of cyclic AMP, and ERK 1/2 activation by Fluorescent Resonance Energy Transfer (FRET) using the EPAC and ERK-NES biosensors, respectively.

Results: The stimulation of PC-3 cells with conditioned media of pre-osteoblasts MC3T3-E1 and osteocytes MLO-Y4 induced an increase in PC-3 adenocarcinoma cell proliferation. Media conditioned by bone cells also caused a transient increase in intracellular calcium accumulation and generation of cyclic AMP and increased ERK 1/2 activation.

Conclusions: Bone cells secrete proliferation-activating factors and signaling pathways that favor the tumor progression of prostate cancer cells, suggesting that cross-communication between these cell types may favor the development of metastatic niches of prostate cancer in the bone.

Key words: *prostate cancer, secreted bone factors, intracellular signaling, fluorescence in living cells, calcium, cyclic AMP, ERK 1/2.*

Introduction

Bone metastasis is a frequent complication in advanced stages of patients with prostate cancer, one of the cancers with greater mortality and morbidity in developed countries[1]. Avoiding the different stages necessary for the tumor cell to abandon the primary tumor, migrate and establish itself in the bone microenvironment is one of the main strategies to prevent bone metastases[2]. The invasion of primary tumor cells into skeletal niches is associated with the activation of bone cells that release growth factors and cytokines, which in turn promote tumor growth in metastases. As a result, the so-called "vicious cycle" of bone metastases is generated, which varies the physiology of bone and alters bone remodeling[3,4]. In the case of bone metastases caused by prostate cancer, osteolytic and osteoblastic lesions are produced as a result of the activation of osteoclasts and osteoblasts respectively[5]. In bone metastasis processes, it has been observed that tumor cells are able to secrete factors such as tumor necrosis factor alpha (TNF-α), interleukin 11 (IL-11), matrix metalloprotease 1 (MMP1), Jagged1 and protein related to parathormone (PTHrP), which directly or indirectly activate osteoclasts, giving rise to osteoclast metastases[6]. Matrix degradation by osteoclasts releases transforming growth factor β (TGF-β) and insulin-like growth factor (IGF-1) that promote the survival of tumor cells[7]. In contrast, the secretion by tumor cells of other factors such as fibroblast growth factor (FGF) and bone morphogenetic proteins (BMPs) can stimulate osteoblast differentiation resulting in osteoblastic lesions[8].

On the other hand, some studies have described the importance of second messengers and intracellular signaling pathways in the modulation of proliferation, malignancy and metastatic capacity of tumor cells. In this way molecules such as calcium, cyclic adenosine monophosphate (cyclic AMP) or kinases regulated by extracellular signals 1/2 (ERK 1/2), have been proposed as mediators and possible therapeutic targets in tumor progression and bone metastasis[9-11].

Despite the existence of various observations analyzing the factors secreted by tumor cells that affect bone cells, there is little information on the factors secreted by osteoblasts and osteocytes that act on tumorigenic prostate cells. In particular, the effect of factors secreted by bone cells on signaling pathways and second relevant messengers in the mediation of processes of tumor progression and metastasis to bone in tumor cells of prostate is little known.

In this study we have used fluorescence techniques in living cells to analyze whether factors secreted by bone cells can modify signaling pathways and second messengers in prostate adenocarcinoma cells. Our observations show that factors secreted by osteoblasts and osteocytes can induce proliferation of prostate tumor cells associated with accumulation of intracellular cyclic AMP and calcium and activation of the ERK kinase. These results suggest the key role of bone factors in intracellular mechanisms relevant to tumor progression and bone metastasis.

Material and methods

Cell cultures

Human prostatic carcinoma cells derived from bone metastases (PC-3, ATCC: CRL-1435) were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS). The murine pre-osteoblastic cell line MC3T3-E1 (ATCC: CRL-2593) and murine osteocytic MLO-Y4 (generously donated by Lynda Bonewald) were cultured in DMEM with 10% FBS or α-MEM with 2.5% fetal serum from Ram (SCF) and 2.5% SFB, respectively. All cells were cultured in media containing penicillin (100 units/mL) and streptomycin (100 µg/mL) in a humidified incubator at 37°C and 5% atmospheric CO_2. Conditioned media were obtained from PC-3, MLO-Y4 or MC3T3-E1 cells cultured in α-MEM in the absence of serum for 24 h.

Transfections

For transient transfections, PC-3 cells were cultured on glass coverslips of 25 mm diameter for 12 h prior to transfection with FuGENE 6 (Roche Applied Science), which was performed in complete culture medium. After 24 h the cover slips were transferred in an Attofluor chamber (Invitrogen, Carlsbad, CA) with HEPES/bovine serum albumin solution (BSA) (pH=7.4) (HEPES 0.1% (w/v) ASB solution) for real-time fluorescence experiments.

Cell proliferation assay

The number of viable PC-3 cells stimulated with conditioned media of cells MC3T3-E1, MLO-Y4 or of the PC-3 itself was evaluated by the trypan blue exclusion test as previously described[12].

Measurement of intracellular calcium

The accumulation of intracellular calcium was quantified with the calcium sensitive sensor Fluo-4/AM (Invitrogen, Carlsbard, CA) following the manufacturer's protocol as previously described[13]. Briefly, PC-3 cells were cultured on MatTek culture plates with 2 µM Fluo-4/AM in Hanks' balanced salt solution (Invitrogen) at 22°C for 45 min. The cells were washed three times in the Hanks' solution and incubated at 22°C for 30 min. The intracellular calcium quantifications were performed with the inverted fluorescence microscope Nikon A1s. The fluorescence levels were measured at intervals of 1 s to 20 min. At least 30-40 cells were evaluated under each condition. The reagents ionomycin (increases the entrance of calcium ions in the cells) 10 µM and EGTA (calcium chelator) 10 mM were used to obtain the maximum and minimum stimulation in each cell analyzed.

Fluorescent Resonance Energy Transfer (FRET): assessment of intracellular

PC-3 cells were transiently transfected with EPAC cyclical AMP biosensor[14] or with the ERK phosphorylation biosensor, ERK-NES[15]. The generation of cyclic AMP and the activation by phosphorylation of ERK were evaluated by Energy Transfer by Fluorescent Resonance (FRET) as previously des-

cribed[16]. The cells were cultured in Ibidi culture plates of 35 mm diameter and kept in FRET buffer solution (137 mM NaCl, 5 mM KCl, 1 mM CaCl 2, 1 mM MgCl 2, 20 mM HEPES, 0.1% bovine serum albumin, pH 7.4) where they were transiently transfected with constructs consisting of the fusion proteins: fluorescent protein cyan (CFP)-EPAC-yellow fluorescent protein (YFP) or by CFP-ERK-NES-YFP and which is activated by direct binding of cyclic AMP or by phosphorylation, respectively, undergoing conformational changes that result in variations in FRET responses. Quantifications were carried out on a Leica microscope equipped with a 40x objective of immersion oil, sequential records of the CFP and YFP fluorescence channels being made. The intensities of the fluorescence emission were determined at 535/15 nm (YFP) and 480/20 nm (CFP) with a long dichroic passage (DCLP) of 505 nm. The FRET signal was monitored as the emission index of YFP (FYFP) and CFP (FCFP). The results are shown as the normalized mean (nFRET) ± standard error.

Statistical analyses

The data were expressed as mean ± standard error. The differences between the experimental conditions and the controls were made using the U Mann Whitney statistical test, in which values of $p<0.05$ were considered significant.

Results

Soluble factors of MC3T3-E1 and MLOY-4 induce increased proliferation in human prostate adenocarcinoma cells PC-3

Previous studies suggest that the bone environment favors the stimulation of prostate cancer cells promoting the establishment of skeletal metastases[17]. To evaluate the effects of factors secreted by bone cells on prostate carcinoma cells, we first analyzed the actions of conditioned media of osteoblasts MC3T3-E1 and osteocytes MLO-Y4 on the proliferation of PC-3 prostate cancer cells. Both the conditioned means of osteoblasts MC3T3-E1 and those of MLO-Y4 were found to induce an increase in the proliferation of PC-3 cells after 3 days of stimulation compared to control conditioned media (of the PC-3 cells themselves) (Figure 1).

Osteoblastic and osteoblastic bone soluble factors induce the formation of cyclic AMP and intracellular calcium release in human prostate adenocarcinoma cells PC-3

Next, the effects of conditioned media of bone cells in the activation of second messengers and signaling pathways related to tumor progression, metastasis and the activation of osteogenic responses[9-11,18] were studied by means of fluorescence techniques in living cells. The conditioned media of osteoblasts MC3T3-E1 and osteocytes MLO-Y4 caused a rapid and transient increase in intracellular calcium concentration in prostate cancer cells PC-3 compared to stimulation with medium conditioned by the PC-3 cells themselves (Figure 2A-C). Similarly, the generation of cyclic AMP detected by FRET was stimulated by conditioned means of osteoblasts and osteocytes (Figure 3A-C). The levels of cyclic AMC did not vary when stimulating the PC-3 cells with conditioned media of PC-3 (data not shown).

Activation of the ERK 1/2 signaling pathway in human prostate adenocarcinoma cells PC-3 after stimulation of soluble bone factors

Phosphorylation of ERK 1/2 kinase, a protein directly involved in the proliferation of prostate tumor cells[19], was also induced by conditioned media of osteoblasts MC3T3-E1 and osteocytes MLO-Y4 (Figure 4A and B). The conditioned medium of PC-3 cells, on the other hand, did not cause changes in the phosphorylation of ERK 1/2 of PC-3 cells (Figure 4B).

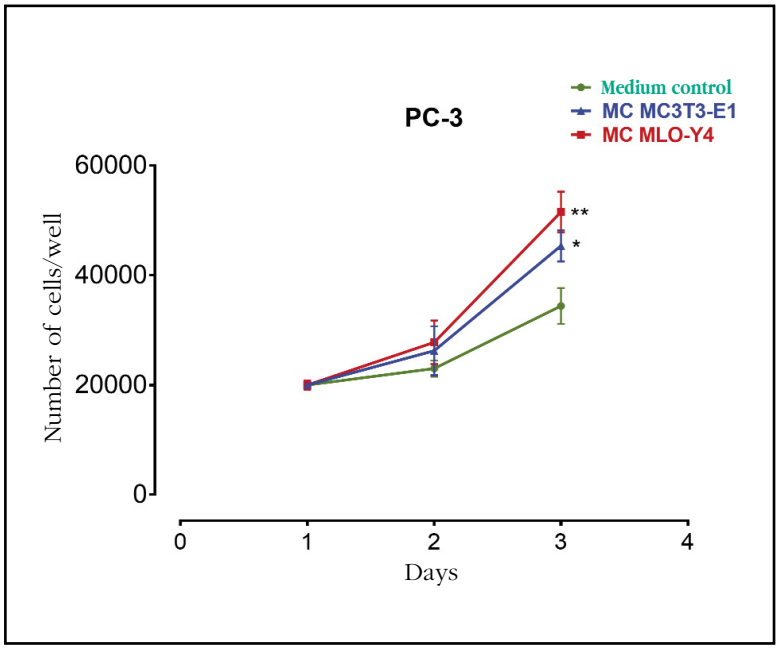
These results as a whole show that the factors secreted by bone cells modulate key signaling molecules in cellular processes such as the proliferation of prostate tumor cells.

Discussion

Our results show that metastatic prostatic adenocarcinoma cells increase their proliferation with factors secreted by both osteoblastic and osteocytic cells. In the case of bone metastases, it has been hypothesized that tumor cells are established in specific areas of bone such as the endosteal niche, the niche of hematopoietic stem cells and the vascular niche[20]. These niches are complex microenvironments in which factors that promote the physiological functions of the cells that compose them are secreted. It has been shown that increasing the number of these niches experimentally also increases the number of disseminated tumor cells of primary tumors[21]. These observations suggest that the same factors that maintain the correct functioning of the cells of the bone niches are able in turn to promote the establishment and growth of tumor cells in bone metastases. From this point of view, osteoblasts and osteocytes located near the surface would form part of the endosteal niche and may generate factors that promote the growth of prostate tumor cells in this niche.

There are several mechanisms that regulate the mitotic cycle of metastatic cells in bone, including regulatory processes of the immune system, angiogenesis, extracellular matrix, various factors and hormones, and intracellular processes[22]. Among these mechanisms, it was observed that the balance in the activation between 2 protein kinases activated by mitogens (MAP kinases), p38 and ERK 1/2 affects in a key way the mitosis of metastatic tumor cells[23]. When ERK 1/2 is activated in comparison with p38, cell proliferation is favored, and on the contrary the activation of p38 against ERK 1/2 induces a cellular quiescent state[23]. We have observed that pre-osteoblasts and osteocytes can send soluble factors that activate the ERK 1/2 kinase in PC-3 cells thus promoting the proliferation of tumor cells.

Figure 1. Factors secreted by osteoblasts MC3T3-E1 and osteocytes MLO-Y4 increase the proliferation of PC-3 prostate carcinoma cells. The PC-3 cells were incubated for 1-3 days with conditioned media (MC) obtained from MC3T3-E1 or MLO-Y4 and the number of cells was evaluated by trypan blue assay. The data shown are means ± standard error of 3 independent experiments *p<0.05; **p <0.01 vs. Conditioned medium (MC) Control



In addition, we have observed that factors secreted into the environment conditioned by pre-osteoblasts and osteocytes also caused a transient increase in intracellular calcium concentration and in the generation of cyclic AMP. Both second messengers can regulate processes of proliferation and tumor metastasis and have been proposed as possible therapeutic targets in several cancers[9,10,24]. Cyclic AMP can have positive or negative effects on the growth and survival of tumor cells depending on the cell type[10]. In tumors of epithelial origin such as prostate cancer, cyclic AMP seems to play a role in promoting oncogenesis by activating protein kinase A and other proteins activated below (for example, EPAC and CREB)[25,26].

On the other hand, it has been shown that the increase in intracellular calcium concentration of extracellular origin is a factor that induces the proliferation of prostate cell lines of bone metastases (PC-3 and C4-2B), but does not affect the proliferation of non-metastatic prostatic cell lines such as LNCaP[9] cells. The increase in the concentration of calcium of extracellular origin causes PC-3 an increase in the expression of cyclin D1 (a regulatory protein of the cell cycle necessary in the proliferation), in the activation of Akt (protein required for the proliferation and tumor progression)[27,28], and increases the binding capacity of tumor cells to substrate[9]. In addition, alterations in the gene expression of various calcium ion channels, such as TRP and Orai, have been associated with increases in calcium entry in prostate tumor cells that facilitate proliferation and resistance to apoptosis of those cells[29,30].

Overall, these studies show the relevant function of the activation of the kinase ERK 1/2, calcium and cyclic AMP in the progression of prostate cancer. Although the modulation of these signaling pathways by factors secreted by bone cells has not been previously described, some studies have demonstrated the ability of resident bone cells to modulate the activity of tumor cells in metastatic niches. It has been observed that osteocytes mechanically stimulated by increased pressure caused by metastatic tumors induce growth and invasiveness of prostate tumors through the secretion of chemokine (C-C) ligand 5 (CCL5)[31]. Interestingly, the stimulation of cells of different types of cancer by CCL5 is able to increase the invasive and migratory capacity of tumor cells through mechanisms dependent on the intracellular mobilization of calcium[32] or activation of the ERK kinase[33,34]. These observations suggest that CCL5 or other similar factors of the secret of bone cells could be responsible for the changes in signaling pathways of tumor cells that we have observed in the present study. On the other hand, previous publications have also demonstrated the key role of bone cells in promoting the activation of tumor cells and favoring metastatic processes based on direct bone cell-tumor cell contact through the activation of the Notch-Jagged signaling pathway[35]. Factors secreted by bone cells may mediate initial metastatic tumor recruitment and growth processes, where there is no direct contact between the tumor and the bone cells, while signaling pathways such as Notch-Jagged may regulate the interactions of the tumor. tumor in more advanced metastatic phases (in which the tumor does come into direct contact with bone cells).

Based on these investigations and our results, we propose that osteoblastic and osteocytic cells regulate the proliferation and activation of molecular mediators of tumor progression in metastatic prostate cancer cells by the secretion of soluble factors. We also suggest that the modulation of calcium intracellular mediators, cyclic AMP and ERK 1/2 by factors secreted by bone cells could be key in the establishment of bone metastases by prostate tumor cells.

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

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Figure 2. Factors secreted by MC3T3-E1 and MLO-Y4 increase the intracellular calcium signaling of PC-3. We analyzed the effects of conditioned secreted factors obtained during 24 hours of MC3T3-E1 (A), MLO-Y4 (B) or PC-3 (C) in the intracellular calcium release of PC-3. The evaluation of intracellular calcium levels was performed by confocal fluorescence in living cells with the Fluo-4AM indicator as described in the text. The arrows indicate the moment of stimulation with conditioned means. The data shown are means ± standard error of 3 independent experiments

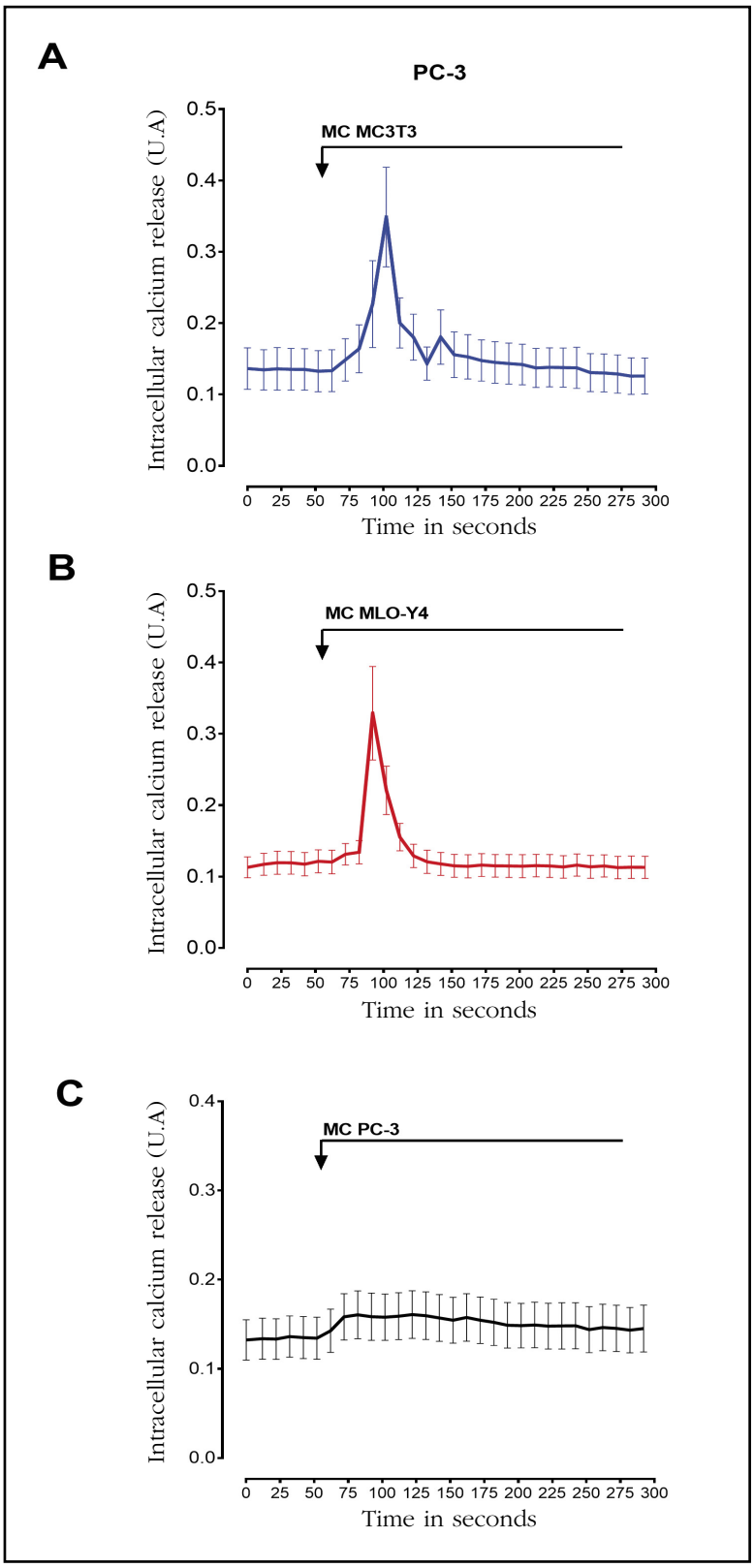

Figure 3. Factors secreted by MC3T3-E1 and MLO-Y4 increase the cyclic AMP signaling of PC-3. (A) We analyzed the effects of conditioned secreted factors obtained during 24 hours of MC3T3-E1 and MLO-Y4 in the activation of PC-3 cyclic AMP. The evaluation of cyclic AMP was performed by confocal fluorescence in living cells with the CFPE-PACYFP sensor as described in the text. The arrows indicate the moment of stimulation with conditioned means. Forskolin was used to obtain maximum stimulation of cyclic AMP. The data shown are means ± standard error of 3 independent experiments. (B and C) Representative images of the fluorescence changes of the CFP and YFP fluorescent proteins of the EPAC cyclic AMP sensor in PC-3 cells after stimulation with conditioned medium of MC3T3-E1 or MLO-Y4 cells

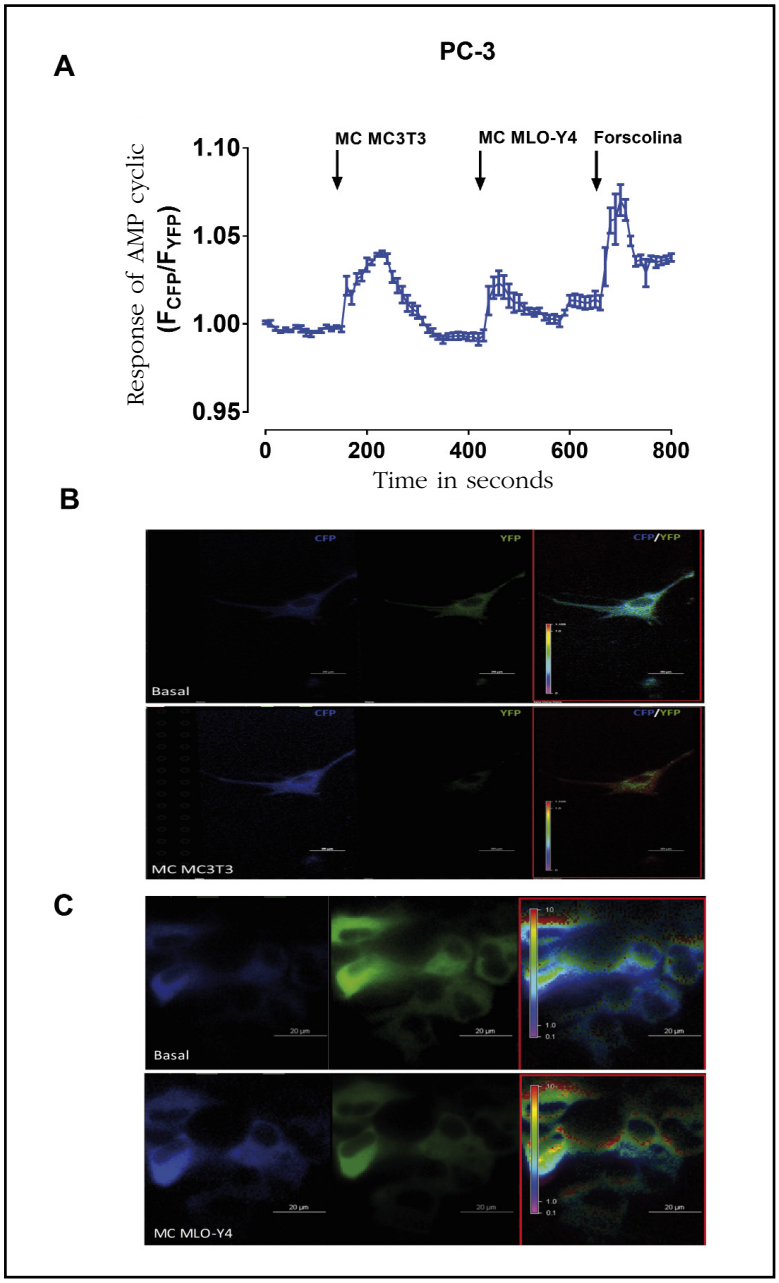

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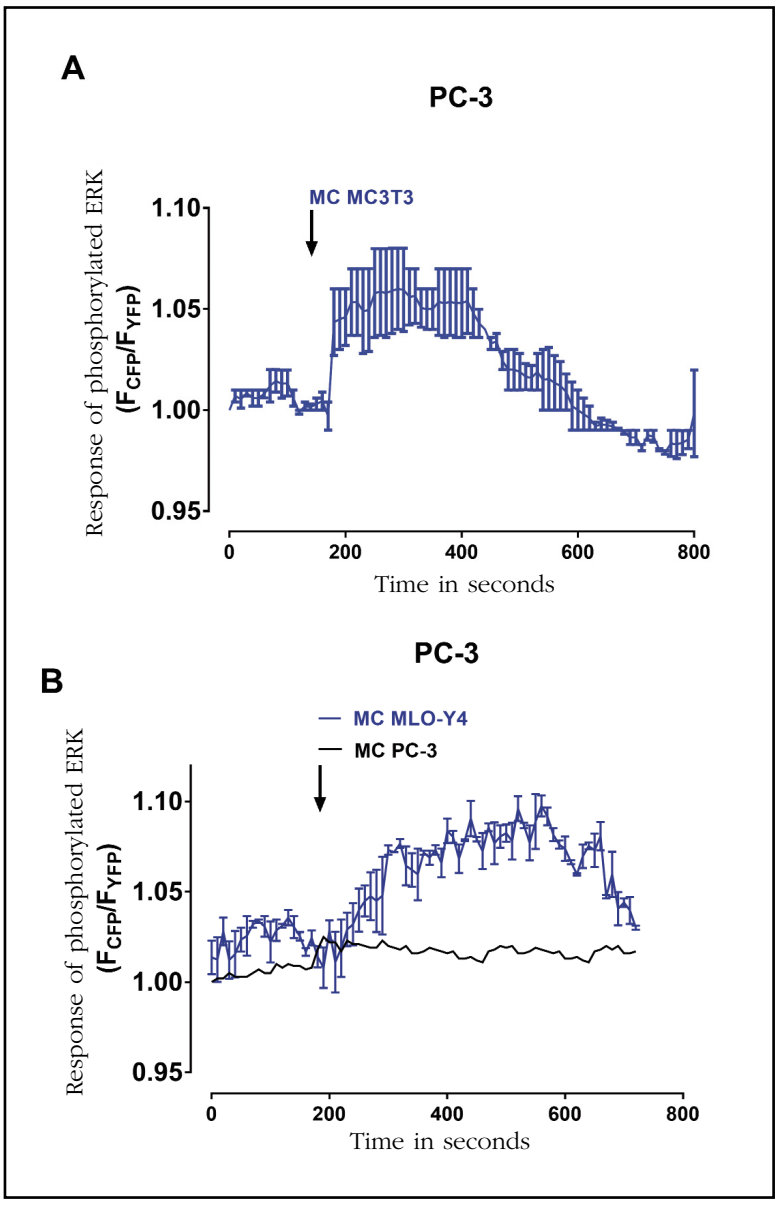
Figure 4. Factors secreted by MC3T3-E1 and MLO-Y4 increase the phosphorylation of the ERK 1/2 kinase of PC-3. We analyzed the effects of conditioned secreted factors obtained during 24 hours of MC3T3-E1 (A) or MLOY-4 (B) on phosphorylation of the ERK 1/2 kinase in PC-3. As a control, PC-3 cells were stimulated with conditioned medium of PC-3 cells. The evaluation of cyclic AMP was performed by confocal fluorescence in living cells with the CFPERK-NESYFP sensor as described in the text. The arrows indicate the moment of stimulation with conditioned means. The data shown are means ± standard error of 3 independent experiments



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Isoflavones and bone health

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Summary
Phytoestrogens are a family of plant-derived components that present a steroid structure and can act in the estrogen receptor. They contain both estrogenic and antiestrogenic properties, depending on the tissue in which they act.
The potential mechanisms by which phytoestrogens can affect cell activities have been divided into genomic and non-genomic effects. The former act through estrogen receptors, and the latter are mediated by cellular proteins. The active mechanism of soy isoflavones in bone may be beneficial, as they act by stimulating the activity of the osteoblasts. On the other hand, through the RANK-L/OPG system they bring about a decrease in osteoclast survival and activity. This article reviews *in vitro* studies, in animals and humans, that involve isoflavones and bone health to ascertain how these substances affect those postmenopausal women who use them in treatment or prevention of the climacteric syndrome.
In general, the global assessment of human studies shows variability in the design, in the variety of isoflavone sources, in the time of the analysis and in the dose. In addition, the variability in the bioavailability and metabolism of isoflavones between the subjects must be considered. All this makes it difficult to obtain consistent conclusions.
To sum up, some positive results justify the need for further research. From a clinical point of view, isoflavones are used in women with climacteric symptoms who cannot or do not wish to undergo hormone therapy. They would not be indicated for treating osteoporosis, but those women who use them at the right doses and time can expect a benefit in maintaining bone mass.

Key words: *bone health, soy, isoflavones.*

Introduction

The estrogenic deficit derived from decreased ovarian function leads to increased bone remodeling, with a negative balance that contributes to a loss of bone mass. The result is the increased risk of developing osteopenia, osteoporosis and, as a consequence, increased risk of fracture.

Hormone therapy is considered a very effective treatment for the relief of climacteric symptoms. It has been shown to have a beneficial effect on bone with reduction of vertebral and hip fracture even in non-osteoporotic postmenopausal women[1], but information about the increased risk of some chronic diseases have markedly increased the interest of clinicians and women for alternatives to this treatment. Some of the most popular are based on food or phytoestrogen supplements.

Of all the natural alternatives currently under study, phytoestrogens and their components, isoflavones, seem to offer the greatest potential for bone loss prevention.

Isoflavones and bone metabolism

Phytoestrogens are a group of plant-derived compounds that have been shown to have both estrogen agonist and antagonist properties, depending on the tissue where they act.

Based on their chemical structure, phytoestrogens are divided into four main classes:

1.- Isoflavones
2.- Stilbenes
3.- Coumestans
4.- Lignans

Isoflavones are the best known, with their main representatives genistein and daidzein. They are found in significant quantities in soybeans. The chemical structure is similar to 17β estradiol and can bind to estrogen receptors (ER). The binding of a phytoestrogen to ER may result in partial activation of the same (agonist effect) or displacement of an estrogen molecule, which reduces receptor activation (antagonistic effect).

They have an affinity for ER that is lower than that of estradiol. The affinity and the time of occupation of the isoflavones by the β receptor is about 30 times higher than by the α receptor. In tissues, there is a different distribution of these receptors, suggesting that they exert selective tissue effects depending on the tissue in which they act. In the reproductive tissue, especially the uterus and breast, the type predominates, while the bone tissue has a greater amount of receptors β[2]. In addition, isoflavones present other actions independent of ER, such as enzymatic inhibition or antioxidant activity.

The exact mechanisms of the effect of isoflavones and other components of soy on bone are still not fully understood. It has been postulated that the main effect would be genomic through ERs, but other non-genomic effects have also been verified[3].

The presence of ER in osteoblastic cells and genistein binding to ER have been demonstrated. The result appears to be increased bone formation by activating osteoblasts through the genomic mechanism involving the activation of the nuclear estrogen receptor. A variety of non-genomic mechanisms have also been described, including the inhibition of tyrosine kinase and topoisomerase II[4]. On the other hand, it has been shown that daidzein induces apoptosis of osteoclasts[5].

Another mechanism of action proposed more recently is through increased osteoprotegerin synthesis (OPG) by the osteoblast. In a cohort of osteopenic postmenopausal women, genistein administration compared with placebo showed that the level of RANK-L was lower ($p<0.001$ vs. placebo) and that of OPG higher ($p<0.001$ vs. placebo) in the follow-up over one and two years[6].

Another possible mechanism of action is the different behavior of soy protein compared to animal proteins versus intestinal calcium absorption[7]. The consumption of soy protein produces a lower urinary calcium excretion than the intake of animal protein[8]. This could have clinical importance regarding recommendations on health habits for postmenopausal women, suggesting the substitution of animal protein for soy protein.

In summary, from the physiological point of view of bone remodeling, these findings support the hypothesis of a stimulating effect of osteoblasts and a possible inhibiting effect of osteoclast recruitment (via RANK-L), as well as a shortening of their half-life to promote its apoptosis. The result would be an antiresorptive effect with positive balance towards the formation mediated by the OPG, and with action in the ER, but also through the action in certain enzymes[9].

In vitro studies

Many basic research studies indicate the positive effect of isoflavones on the variables related to bone metabolism, osteoporosis, fracture and bone quality. The MC3T3-E1 osteoblastic cells have been cultured in medium containing various concentrations of daidzein, showing a significant increase in alkaline phosphatase activity and protein content. This effect is completely counteracted by adding an antiestrogen such as tamoxifen, which indicates the stimulatory effect on proliferating and differentiating the osteoblastic cells MC3T3-E1 mediated through the ER. The effect of genistein on osteoblastic cells seems to be the same as that of daidzein[10].

On the other hand, genistein inhibits osteoclast activity directly through tyrosine kinase inhibition, which in turn inhibits bone resorption[11].

Studies in animals

Most studies of phytoestrogens action on bone, in animal experiments, have been carried out in ovariectomized rat (OVX) models and some in primates. They vary considerably depending on whether the administration route has been subcutaneous, continuous parenteral injection or oral feeding. In general, the analyzed product is isoflavones, either pure compounds (mainly genistein) or soy proteins, with or without their isoflavones, but with a wide variety of doses used.

Controls have been made with casein or semipurified diets. In several studies, the effect of phytoestrogens has been compared with conjugated equine estrogen or estradiol.

The main objectives have been the variation of the trabecular and/or cortical bone mass, bone mineral density (BMD) and the mechanical resistance in some studies. Secondary objectives included variation in markers of bone turnover and effects on uterine weight.

In general, the effects of isoflavones in the skeletal tissue of experimental animals have been consistent in the sense of showing a favorable effect of isoflavones on bone.

The first studies examined the effects of soybean milk[12] and soybean protein[7] compared to casein in the animal model of OVX rats. The rats fed soy diet showed significantly higher bone density in the femur and lumbar spine than the rats in the control group. The question of whether the effect was due to the protein itself or to the presence of isoflavones in soy is not clarified in these initial studies. To clarify this question, the OVX rats were fed a diet containing 44 µmol/day of genistein. The control rats were fed an identical diet in which the isoflavones were eliminated. The results showed that genistein was effective in reducing bone loss in OVX rats, supporting the hypothesis that it would act as an osteoclast inhibitor[4].

In another study, soy protein isolate proved to be as effective as estradiol in controlling bone loss after ovariectomy was carried out in rats[13].

However, in another study, which showed a significant IGF-1 mRNA increase in the groups treated with isoflavone, and in a dose-dependent way, no significant effect on bone density was found[14].

Using techniques such as DXA, the trabecular bone volume of the distal femoral metaphysis was reported to be markedly reduced in OVX mice, showing the genistein capacity to restore this loss[15].

In a randomized trial that studied the ability to reverse bone loss already established by daily intake of soy isoflavones in the long term and in different doses (20, 40 or 80 mg/kg/day for 84 days), and carried out in rats from which ovaries were removed and rats that underwent simulated surgery conserving the ovaries[16], the BMD was significantly lower in the OVX rats than in those that underwent sham surgery. Feeding with isoflavones did not affect BMD in this population. Neither induced changes in uterine weight, indicating the absence of uterotrophic effect. The anti-osteoclastic activity induced by isoflavones occurred in a dose-dependent manner. However, although isoflavone administration reduced bone turnover, it did not reverse the already established bone loss. These results support the idea that consumption of soy isoflavones may have a more preventive than curative role in bone health.

The importance of neonatal exposure to isoflavones has been reported. The analysis of BMD and bone resistance in mice in adulthood is higher when they have had an intrauterine exposure to genistein and/or daidzein[17].

In bone quality analysis, genistein retained the biomechanical quality of trabecular bone regardless of microstructure parameters, such as density or length of microfractures, mineral apposition rate or BMD[18].

However, studies in primates do not concur with results in OVX rats. In premenopausal *cynomolgus* monkeys (*Macaca fascicularis*), a high-isoflavon content soybean diet did not significantly affect bone characteristics, BMD, or bone biomarker measurements[19]. In ovariectomized monkeys, no effect of soy phytoestrogens in the diet was observed for any bone mass measurement[20], and soy protein alone did not prevent the increase in bone turnover[21].

In summary, the effect of isoflavones in basic research (*in vitro* studies and animal models) points to:

- Reduction of markers of bone resorption
- Increase in markers of bone formation
- Preservation of bone structure and quality
- Preservation of bone resistance to fracture

Human studies

Observational studies

Observational studies of dietary intervention have shown similar findings to the *in vitro* effects of phytoestrogens in bone cell cultures and markers of bone turnover, which are indirectly consistent with the reduction of bone remodeling.

Most observational studies on bone markers have been conducted in women living in countries where the population has a relatively high intake of phytoestrogens. These have found a significant inverse correlation between isoflavone intake and urinary excretion of bone resorption markers pyridinoline and deoxypyridinoline in postmenopausal women of Asian countries[22].

Among Asian populations, several observational studies show that postmenopausal women who consume soy foods, and therefore isoflavones, present the highest BMD of the lumbar and/or hip spine[23], as in American populations of Japanese origin[24,25]. A greater peak of bone mass and the maintenance of this bone mass in young women has been described[26], and a lower loss in perimenopausal women[27] and postmenopausal women[28]. This effect has not been shown in breast cancer survivors[29].

In adults who live in Western countries, the data are limited, so it is difficult to draw conclusions about the relationship between phytoestrogen intake and BMD or the rate of fractures, since their consumption is generally insignificant in these countries[30]. A study in American white women found a decrease of 18% in resorption markers in those with high intake of genistein in the diet[28].

Clinical studies

The biggest problem with clinical trials in humans that analyze the effect of isoflavones on bone is the great variability in terms of design, source of the products analyzed, dosage and, especially, the relatively short duration in order to accurately

detect significant changes in the BMD. In addition, a confounding factor in isoflavone treatment studies is the variability in the bioavailability and metabolism of isoflavones among subjects.

Subjects vary from low to moderate and high metabolizers. Therefore, even if the same dose of isoflavone is administered, a variability in response can be expected. Daidzein is metabolized to equol by the gut microbiota in approximately 30% of people. This metabolite is biologically more active than its precursor[31].

A one-year randomized, double-blind, placebo-controlled trial administering equol supplements (10 mg/day) to 93 non-equol menopausal Japanese menopausal women showed that the intervention increased the concentrations of this metabolite in serum and urine in a dose-dependent manner. Urinary deoxypyridinoline decreased significantly, with a -23.94% change in the group receiving equol supplement compared to a change of -2.87% in the placebo group (p=0.020). In addition, BMD was maintained in the treated group, which decreased in the placebo group[32].

There are few reports of studies in premenopausal women, and it is not possible to draw conclusions about the impact of phytoestrogens on bone in them[33,34]. The administration of soy rich in isoflavones had no effect on BMD in healthy young adult women with normal menstruation[35].

In another 24-week study conducted in 69 perimenopausal women, the effect on bone loss of administering soy protein rich in isoflavones (80.4 mg/day), soy protein poor in isoflavones or casein (4.4 mg/day) was analyzed. The control group had a significant loss of bone, while the treatment rich in isoflavones attenuated lumbar spine bone loss[27]. A study of similar design in postmenopausal hypercholesterolemic women obtained similar results[36].

According to this information, it is possible that the inclusion of soy products containing isoflavones in diets of perimenopausal women can attenuate bone loss and decrease the risk of osteoporosis. However, in another study conducted in apparently healthy early postmenopausal white women (51-56 years), consumption of foods containing soybean isoflavone aglycone at 110 mg/day for one year did not prevent postmenopausal bone loss nor did it affect bone turnover[37]. Similar results are shown in another study in late postmenopausal women, with one year of follow-up, in which, although changes were identified in the markers, it did not occur in BMD, even when the results were analyzed by producers and non-producers of equol[38].

The cooperative effects of isoflavones and exercise on bone and lipid metabolism were analyzed in 128 postmenopausal women during 24 weeks randomly assigned to 4 groups: placebo; placebo combined with walking (3 times a week); Isoflavone intake (75 mg of isoflavone conjugates per day); and isoflavone combined with walking. The combination of isoflavones and exercise showed favorable effects on serum lipids and body composition of postmenopausal women. The findings of this study suggest that the preventive effects of isoflavones on bone loss depend on the individual's intestinal microbiota for the production of equol[39], although other studies do not find differences depending on the producer or non-producer phenotype of equol[37].

A meta-analysis of ten randomized, placebo-controlled trials, related to the effects of soy isoflavone intake on BMD of the lumbar spine, included 608 women who were administered soy isoflavones in doses of 44-160 mg/day with a treatment time of 4-24 months. In conclusion, the intervention with isoflavones significantly attenuated the bone loss of the spine in menopausal women. These favorable effects are more marked when more than 90 mg/day of isoflavones are administered. The beneficial effect would be evident after consumption for 6 months[40].

However, another meta-analysis that included ten randomized, placebo-controlled trials of at least one year and that analyzed 896 women indicated that supplementation with soy isoflavones is unlikely to have a significant favorable effect on BMD. in the lumbar spine and the hip. Similar results were obtained in subgroup analyses by sources of isoflavones (soy protein vs. isoflavone extract) and ethnic differences (Asian vs. western). Only the analysis according to the dose equal to or greater than 80 mg/day compared to lower doses tended to have a weak beneficial effect on the BMD of the lumbar spine[41].

On the effects of isoflavone intake on markers of bone remodeling, the results of nine randomized trials in which 432 subjects were included in a meta-analysis. It was concluded that the intervention with isoflavones significantly inhibits resorption and stimulates bone formation, according to the response of bone turnover markers. These favorable effects occur even if <90 mg/day of isoflavones are consumed or if the intervention lasts less than 12 weeks[42].

The effects of isoflavones on bone strength in humans are unknown. It has been indicated that the treatment with soy isoflavones over 3 years was modestly beneficial in the measurement of the volumetric bone mineral density of the medial femur, as well as in the force-deformation index[43].

A recent meta-analysis[44] analyzed the effect of isoflavones on BMD. We included 21 studies with 2,652 postmenopausal women. The results indicated that in the lumbar spine, treatment with isoflavones is associated with a significant increase in BMD compared to the control. In the femoral neck, the number of studies that provide this information is 18 (n=1,604), also finding a significant change. The studies that used isoflavones aglycone found better results compared to the control, being higher the effects to the studies that used the glycosylated forms.

Genistein reduced the urinary excretion of pyridoline and deoxypyridoline by increasing the levels of alkaline phosphatase and insulin-like growth factor-1 (IGF-1), without showing changes

in the ultrasound measurement of the endometrial thickness. The authors concluded that treatment with isoflavones exerts a moderate beneficial effect against bone loss related to estrogen deprivation in post-menopause. The effect seems to be related to the aglycone form of the isoflavones.

Effect on fracture risk

The only information on the effect of isoflavones on fracture risk is derived from some population studies. There are no clinical trial data on fracture.

A prospective study of a large Asian cohort[45] of 24,403 postmenopausal women with no history of fracture or cancer, followed for a mean of 4.5 years, and after adjusting for age, socioeconomic status, osteoporosis risk factors, and other dietary factors, found a relationship of fracture risk with the consumption of soy protein or isoflavones, with an inverse relationship that was more pronounced in women in early menopause. The authors concluded that soy consumption can reduce the risk of fracture in postmenopausal women, especially among those approaching menopause.

Conclusions

Evidence from epidemiological and prospective cohort studies indicates a positive effect of isoflavone intake on the risk of osteoporosis and fragility fracture.

There are several mechanisms of action that explain the actions of isoflavones on bone and, although the exact mechanisms involved are not fully understood, it seems that the consumption of soy isoflavones attenuates bone loss induced by menopause by decreasing resorption and training stimulation.

As shown consistently in both *in vitro* and animal studies, isoflavones appear to stimulate bone formation through action on osteoblasts, being able to inhibit bone resorption by acting on osteoclasts and thus establishing a positive balance

Human studies show variability in the results due, at least in part, to the different methodology used, the variety of isoflavone sources, the doses used and the time of the analysis; to which we must add the variability of the bioavailability and the metabolism of the isoflavones between the subjects, being sometimes difficult to separate the results of a possible genetic and environmental influence.

The studies reviewed show evidence of a beneficial effect of soy isoflavones on bone health in perimenopausal and postmenopausal women when soy protein with high isoflavone content is incorporated into the diet. This could be an adequate strategy to improve the bone health of postmenopausal women.

The evidence is insufficient to recommend the consumption of isoflavones for the prevention or treatment of osteoporosis, but in those women taking adequate doses of isoflavones, lower BMD loss related to estrogenic deprivation can be expected.

The results of the studies show some positive results, which justifies the need to carry out additional clinical trials in which it would be desirable to have a larger sample population and a longer duration than allowed, in addition to demonstrating the effect of isoflavones on the biochemical markers of bone remodeling, bone density and bone quality, investigate the effect on the prevention of fractures.

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

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A proposal for reorganizing the world of scientific publications which would save Spain millions of euros

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1. What was the reason for the scientific publications? How everything changed with Eugene Garfield

At the beginning of the twentieth century few scientific journals existed and their range of diffusion was limited. In the field of medicine, two publications stood out: in the United States, The New England Journal of Medicine, which was established in 1812, and The Lancet in Europe, which dates back to 1823. The main objective of the authors, most of whom were researchers, was to report important findings to their scientific community. These findings were often expected, as, for example, with Watson and Crick's publication of the breakthrough in the structure of DNA in Nature[1] or Fleming's discovery of penicillin[2], milestones in medicine that became known through their publication as scientific articles or simply as a letter, as in the case of the discovery of DNA[1].

More than 30 years ago, everything changed. Eugene Garfield's impact factor for scientific journals[3] was initially conceived as an index to assess the quality of journals and to provide orientation for librarians (the essence of the impact factor is to list the frequency with which a given article is cited in other quality journals as well as the number of articles that the magazine publishes)[4]. The impact factor suffered a malevolent distortion in its use and, by extension, began to be used as an index of quality of the scientific articles published in the journals with an impact factor. From that point on, it conditioned the professional attitude of publishers, scientific journals, researchers and even of research institutes, universities and ministries, a phenomenon that has been recognized and lately called into question[5].

The impact factor immediately led to the division of journals into the "first category" ones which were those included in the Journal of Citation Reports (JCR), and all the others, which were not and, therefore, did not have this impact factor. In turn, the journals included in JCR were classified in quartile rankings, where the best journals were those at the top of this list. This fact conditioned the development of a chain, whose assertions, erroneous, in our opinion, carry on until our present day: journals with the greatest impact are best, the best articles are published in the most impacting journals. So, that is why they are the best. The best researchers, those who produce the highest quality articles, publish them in the journals with the greatest impact. So, the best way to assess the quality of a research study (of its researchers, its research institutes, its hospitals, etc.) is assessing the impact factor of its publications (which is, really, that of the journals where they are published). For this reason, depending on the impact factor, grants and subsidies are bestowed for research, scholarships, research appointments, and even assessments by the evaluation agencies for the accreditation of university professors, professors and holders. Everything revolves around

the impact factor and the articles published by the journals that have it. If you have a high cumulative impact factor, you are good at everything. If you do not have it, you do not deserve anything.

2. What is currently the reason for scientific publications?

Disentangle ourselves or accept reality without falsehoods. The main raison d`etre, nowadays, of scientific publications is not to transmit some knowledge to the scientific community. It is true that many of them fulfill this function, but in our opinion, this is secondary and if it were to do so, many journals would be left out. Researcher today more than ever needs to "publish or die"[6-8]. They have entered a vortex from which it is impossible to escape: you need to publish to progress professionally (chairs, entitlements, service heads), to improve our economic conditions (a tenure bonus), to be able to maintain work (scholarships, research grants) and, why not say it, to obtain recognition in the scientific community, which, in addition to improving our *curriculum vitae*, stroke our ego, since a significant number of scientific publications in journals with a high impact factor produce recognition which can lead to invitations to congresses, scientific meetings, advice on new research projects among other perks. Scientific publication has now become a means to achieve other things, to meet needs and self-promotion, whether personal or collective.

3. The business that has developed around scientific publications. Internet came and "with it came the scandal"

With the advent of the Internet, in the final years of the past century, there was a real revolution in scientific publications. Authors could email their articles and then specific editing programs made it possible to significantly shorten the publication process. In addition, the journals could already publish their articles "online." Little by little they would all add a digital edition to their traditional paper format, which still exists today, at least in the most prestigious ones: The New England Journal of Medicine, The Lancet, Nature, Science, Annals of Internal Medicine, The American Journal of Medicine, just to name a few in the field of Internal Medicine. The same has happened in our country with *Revista Clínica Española* or *Medicina Clínica*. But along with these "classic" magazines, a whole new world of publications has been developed with two common elements: they are all digital (that is, they are only published "online", they do not have a paper version). Furthermore, they have adhered to the open access format. This means that readers have full, free access to articles published by journals that have joined this movement. Some of these magazines in this new format have garnered remarkable prestige, displacing even classic journals with "pedigree and breeding". Thus, for example, PLOS One has attained a significant impact factor that places it in the first quartile in internal medicine. But now the maintenance of this method of publishing scientific articles is supported by researchers, **who have to pay** to have their articles published.

Indeed, we have reached a point in this world of scientific publications in which, if we want to publish an article we must choose between a "classic" magazine that is published in traditional format on paper and "online" that does not charge authors, but that charges access to readers, either by personal subscriptions or institutions, or a magazine only "online" in open access format, to which all readers can freely access, but as the author you must pay a significant sum of money. We have gone from "publish or perish" to "pay to publish" (and if not, perish anyway).

4. The daily invitations to publish in these magazines. The fraud that has been generated around them. And who foots the bill?

All those of us who have published an article in a journal with impact factor in the past 5 years are continuously receiving emails from new journals that have just been created. These messages all announce that "they frankly admire our previous article" (from which they obtained our email contact address), they invite us to send them an update or a new version of it. Finally, they inform us that the publication process will be very short, even in less than a month in some invitations with a cost that is never less than € 1,500. Sometimes, the invitation generously includes the invitation to join the journal's editorial committee, which is usually not indexed in Scopus, nor in Google Scholar and much less in JCR, although that there are some exceptions in this regard. Some of these journals try to deceive their potential clients by calculating their own "impact factor", which is not the one obtained in JCR, because it is not included in it, but calculating themselves from Google Scholar, explained with an asterisk and almost illegible fine print, at the end.

But as this matter is all about publishing at any cost (as it were), the end result is that the business that all these journals have created is financed by researchers, immersed in their vertiginous circle of having to publish in order to compete. Most of this money comes from public bodies: universities, research institutes, regional health services, hospitals, foundations, etc., who have had to include in their budgets new items that include the payment of research-generated articles. In a tortuous way, public agencies and health institutions are keeping all these scientific journals and the publishers that are behind this business with the tax money, either by paying for the articles of their researchers, or paying journal subscriptions for their libraries, which are not exactly inexpensive[8]. In one way or another, publishers always win because their business will always be financed by public funds.

In other words, public agencies fund researchers and the research they produce, either directly or as scholarships or grants. To publish the result of this research, you must pay a magazine, which either charges for doing it and then

allows it to be read in open format (open access) or does not charge to publish it but does so in order to read it in subscription form. In one way or another, all public bodies and researchers are working for publishers.

Finally, we must not forget that case of fraud have occasionally been detected. These are non-existent journals, as the investigators afterwards verify the payment and get nothing in return.

A proposal that would save Spain millions of euros

We propose the creation of a Spanish scientific journal, which publishes its scientific articles in Spanish and English exclusively in an ecologically sound digital version constituted by an editorial team of recognized prestige. This would entail the collaboration of qualified and accredited reviewers, for which they could be economically rewarded. This editorial team would have to ensure the veracity and quality of the articles published in order to acquire a scientific prestige from the day one.

The journal would be completely free for authors and readers. That is, the publication of the articles and their access once published would be totally free. Thus, the journal should be publicly financed and edited by a prestigious entity, be it a Ministry or a Research Institute.

The creation, financing and start-up of the journal must be completed with a national agreement at all levels of public, central and regional administrations, so that the articles published in this digital magazine are duly considered in all the sections that we have listed throughout this article: accreditation by state and regional agencies, foundations, universities, regional health services, etc. This is essential, since, in this way, Spanish authors would already feel motivated to send their quality articles to the journal and the cost of maintaining a digital publication of these characteristics would not exceed one month the amount that public institutions pay for 3 or 4 publications in "impact" journals in open access.

The annual amount saved throughout Spain would be several million euros. Isn't this worth trying?

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Review of the scientific evidence regarding clinical use of the Trabecular Bone Score (TBS)

SEIOMM official position (2018)

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Introduction

The incorporation of new technological applications in the medical field entails a prolonged period of evaluation of the scientific evidence generated in the clinical validation process.

Over the past 5 years, numerous publications, communications in congresses and meetings of scientific societies have been generated. The application of the Trabecular Bone Score (TBS) has also received the attention of the International Society for Clinical Densitometry (ISCD), which has integrated it into its official positions.

The concept of Evidence-Based Medicine (EBM) was developed by a group of internists and clinical epidemiologists led by Gordon Guyatt of McMaster University School of Medicine in Canada. The concept of EBM was defined by its creators as the conscious, explicit and judicious use of the best available clinical evidence to make decisions about the care of individual patients. In essence, EBM aims to have the best available scientific information, the evidence, to apply it to clinical practice.

In 2014, the Spanish Society of Bone Research and Mineral Metabolism (SEIOMM) began a project that facilitated its partners TBS software assessment, through a competitive call. The project ended in 2017. This application requires densitometry images with DXA (Dual X-ray Absorptiometry) of the lumbar spine, and by analyzing the image texture, offers information related to the microstructural quality of the trabecular bone. The project had the logistical support of Medimaps, a French developer, which distributed 20 TBS licenses among the partners that proposed their use in certain clinical and therapeutic settings.

Simultaneously, the diagnostic performance in predicting fractures in subjects with decreased bone density, the identification of those who have suffered bone fractures and the evaluation of this new parameter in patient follow-up was assessed.

In order for SEIOMM to achieve a global positioning that it can share with its partners, several experts of the Society have carried out a critical review of the existing scientific evidence on the clinical application of TBS, which is presented here.

Depending on the scientific rigor of the studies' design, their quality is assessed using scales of hierarchical classification of the evidence, from which recommendations are established regarding the adoption of a specific medical procedure or health intervention. All of them have common features. In this case we have used the one used by the Scottish Intercollegiate Guidelines Network (SIGN), since the one proposed by the Agency of Evaluation of Medical Technology (*Agència d'Avaluació de Tecnologia Mèdica* -AATM-) of the Generalitat of Catalonia, which also takes into account the design of the studies, the specific assessment of their quality, requires a volume of scientific evidence created over a longer period of time that allows for producing more publications.

The first work describing the technique and its clinical use date back to 2009-2010. Not until 2013 was there a noticeable increase in the penetration of the new technique and the description of its results (Table 1).

Table 1. Number of publications describing the technique and its clinical use from 2008 to 2017 (provided by courtesy of Medimaps)

Year	Number of publications	Accumulated number of publications
2017	107	290
2016	76	183
2015	39	107
2014	33	68
2013	20	35
2012	6	15
2011	5	9
2010	2	4
2009	1	2
2008	1	1

Interest in this new application for evaluation of the DXA technique for microstructural quality estimation of trabecular bone has experienced an exponential increase, as can be seen in the publication quality graph (Figure 1).

The main international scientific institutions (the American Society for Bone and Mineral Research -The American Society for Bone and Mineral Research [ASBMR]-, the International Osteoporosis Foundation -International Osteoporosis Foundation [IOF]-, the ISCD) dedicated to the field of Metabolic osteopathies and especially the clinical management of osteoporosis have been the main destination of the presentations and publications on the TBS (Figure 2).

The experts' evaluation proposed by the SEIOMM has followed the methodological criteria of the SIGN scale (Table 2), which indicates the level of quality of the scientific evidence and the degree of recommendation that according to it is offered to the readers. A selection of the main publications related to the clinical aspects in which the TBS can influence has been carried out.

The document divides the review process to address three major issues:

1. Can TBS be used to assess the risk of fracture in clinical practice?

2. Can TBS be used to monitor patients with osteoporosis?

3. In what diseases is TBS especially useful?

The SEIOMM experts who have carried out the review of the scientific evidence are Dr. María José Montoya, Dr. José Manuel Olmos Martínez and Dr. Manuel Muñoz, coordinated by Dr. Luis del Río.

Designated issues and reviewers

Reviewer: Dr. José Manuel Olmos Martínez

1. Question: Can TBS be used to assess the risk of fracture in clinical practice?

Proposal of statement 1: The TBS can be used to assess the risk of vertebral fracture, femur and global frailty in women and men from 50 years.

Proposal for statement 2: TBS can be used together with bone mineral density (BMD) to assess vertebral, femur and global fragility in men and women from 50 years of age.

Reviewer: Dr. Mª José Montoya

2. Question: Can TBS be used to monitor patients with osteoporosis?

Proposal for statement 1: The TBS can be used to evaluate changes over time.

Proposal for statement 2: The TBS can be used to assess the effects of treatment over time.

Reviewer: Dr. Manuel Muñoz

3. Question: In what diseases is TBS especially useful?

Proposal for statement 1: The TBS can be used to assess the risk of fracture in subjects with diabetes.

Proposal for statement 2: The TBS can be used to assess the risk of fracture in subjects treated with glucocorticoids.

Proposal for statement 3: The TBS can be used for the clinical orientation of subjects suffering from hypo and hyperparathyroidism.

Proposal for statement 4: TBS can be used for the diagnostic orientation of patients in the presence of osteoarthritis.

1. Question: Can TBS be used to assess the risk of fracture in clinical practice?

Proposal of statement 1: The TBS can be used to assess the risk of vertebral fracture, femur and global frailty in women and men from 50 years.

- Level of evidence, 2++.
- Degree of recommendation, B.

Summary: In 2013, Leslie et al.[1] conducted a retrospective study of a cohort of 29,407 women over 49 years of age in which they assessed the relationships between TBS and the main clinical risk factors for osteoporosis. These authors, using linear regression and multiple regression models, demonstrated that the existence of a low TBS was associated with the recent use of glucocorticoids, a history of previous major fractures, rheumatoid arthritis, chronic obstructive pulmonary disease, high alcohol consumption and an index of high body mass. In contrast, recent therapy against osteoporosis was associated with a significantly lower probability of having a reduced TBS. Therefore, the authors concluded that TBS was strongly associated with many of the predictive risk factors for osteoporotic fractures, which in turn are incorporated into the WHO FRAX® tool. (The FRAX® tool includes the following clinical

risk factors: body mass index (BMI), previous fracture, chronic obstructive pulmonary disease (smoking), use of glucocorticoids >90 days, rheumatoid arthritis, secondary osteoporosis and high alcohol consumption). More recently, McCloskey et al.[2], after monitoring a cohort of 33,352 women aged 40-99 years from the Canadian province of Manitoba, found that TBS remained a statistically significant predictor of major osteoporotic fractures, excluding fracture. of hip (hazard ratio/standard deviation -HR/DE- =1.18 [95% CI: 1.12-1.24]), death (HR/SD=1.20 [95% CI: 1.14-1,26]) and hip fracture (HR/SD=1.23 [95% CI: 1.09-1.38]) after complete adjustment for the risk factors included in FRAX®. These authors[3], in a meta-analysis in which they evaluated 17,809 women and men from 14 prospective cohorts, showed that, after adjusting for the absolute risk of fracture to 10 years provided by the FRAX® tool, TBS continued to act as a risk factor independent of fracture, both main and hip.

Proposal for statement 2: The TBS can be used together with the BMD by area (BMD) to assess vertebral, femur and global fragility in men and women from 50 years of age.

- Level of evidence, 2++.
- Degree of recommendation, B.

Summary: In a retrospective case-control study that assessed the diagnostic performance (sensitivity and specificity) of TBS, BMD and both techniques[4], it was shown that the presence of low TBS and BMD was associated with fractures. in a more powerful way than when only the BMDa is decreased. Thus, the area under the curve (AUC) obtained from the ROC curves was in the first case (TBS and low BMD) 0.732 compared to 0.614 (p=0.005) when only the BMD was low, with the odds ratio (OR) of 2.49 (95% CI: 1.86-3.47) versus 1.54 (95% CI: 1.17-2.03), respectively. On the other hand, del Río et al.[5] found that the combination of TBS and BMD in the lumbar spine improved the prediction of fracture risk in the upper third of the femur. These authors also found that, after adjusting for age, lumbar BMD and TBS maintained their ability to significantly discriminate transcervical fractures (OR=1.94 [95% CI: 1.35-2.79]; 71 [95% CI: 1.15-2.55]), respectively. On the other hand, Leib et al.[6] have obtained consistent results in a larger cohort of Caucasian non-Hispanic American women (n=2,165). In fact, after adjusting for age, weight, BMD, smoking, and family and maternal fracture history, TBS remained a significant predictor of fracture, with an OR of 1.28 (95% CI: 1.13-1.46). The model that combines TBS and BMD increased the association with the fracture by 10%, as expressed by an increase in probabilities of 38% (OR=1.38 (95% CI: 1.23-1.55)). In another study carried out in a small number of women, the combination of TBS and lumbar spine BMD (OR=2.39 [95% CI: 1.70-3.37]) improved the prediction of fracture risk by 25%. Hans et al.[7] showed that the combination of BMD measurement in any region of interest (lumbar spine, femoral neck or total hip) with TBS significantly improved the prediction of fractures compared to BMD or TBS alone (p<0.0001). Briot et al.[8] finally showed that, for the prediction of vertebral fractures, the combination of TBS and BMD of the lumbar spine increased performance in relation to the isolated use of BMD in the lumbar spine (Net Reclassification Improvement -NRI- =8.6%, p=0.046). Therefore, the determination of TBS has recently been incorporated into the factors used by the FRAX® tool to calculate the risk of osteoporotic fracture, which seems to improve the predictive capacity of this instrument for assessing the absolute risk of fracture[9].

2. Question: Can TBS be used to monitor patients with osteoporosis?

Proposal for statement 1: The TBS can be used to evaluate changes over time.

- Level of evidence, 2+.
- Degree of recommendation, C.

Proposal for statement 2: TBS can be used to assess the effects of treatment over time.

After the review of the evidence the proposal of the statement 2: TBS does not improve the monitoring of BMD in the assessment of treatment effects over time.

- Level of evidence, 2++.
- Degree of recommendation, B.

Summary: For a measurement method to be useful in the follow-up of patients, it must be precise and changes influenced by a pathological situation or derived by a treatment must be equal to or greater than minimum significant change (MSC). Several studies have evaluated the accuracy of TBS measurements and have been compared to BMD measurements in the same DXA measurement systems. The first study concerning TBS accuracy was carried out by Hans et al.[7] who evaluated 92 patients from the Manitoba study database, including women aged ≥50 years (51 performed on the same day and 41 remaining carried out after 28 days). The measurement's precision was good with a coefficient of variation of 2.1%. Five other studies found similar results[8,10-13]. In general, TBS accuracy (1.1-2.1%) was comparable to the accuracy of BMD measurements (0.9-1.7%), and there were no significant differences between the different DXA devices. With a confidence interval of 95%, the MSC of TBS is 3.0-5.8%. All these studies included only women. In a more recent study by Krueger et al.[14] a large number of men were included and similar results were found. Of 90 women and 90 men evaluated in a GE-Lunar iDXA by 3 different operators, the same day accuracy was 1.4% for TBS and 1.9% for BMD of the lumbar spine, without significant differences between sexes.

In addition to good accuracy, a useful measure in the follow-up of patients with treatment or derived from the pathological situation requires that the change be of sufficient magnitude to be detected. Several cross-sectional studies have shown a significant decrease in TBS with age.

In a study of 5,942 French Caucasian women[11] a linear decrease of 14.5% was found in TBS between 45 and 85 years of age. 8.5% of this loss occurred after 65 years of age. Similarly, a 16% decrease in TBS was observed in 619 Caucasian women between 45 and 90 years old[15]. In a study of 3,069 Japanese women aged 45-80 years, a 19% decrease in TBS was detected[16]. In 518 African-American women aged 50-80 years, there was a less pronounced decrease in TBS of 4.6%[17]. The most important longitudinal study based on the Manitoba database sample size found a significant decrease of 0.31±0.06% per year in the TBS during an average follow-up of 3.7 years, similar to the decrease of 0.36±0.05% per year observed in the BMD of the lumbar spine in untreated patients[18].

Currently, there are several types of effective and safe drugs for the treatment of osteoporosis and in the studies reviewed in preparation of this paper, one or more of these treatments were evaluated. The TBS has been analyzed in 12 studies in patients treated with bisphosphonates, in 5 of them with denosumab, in 7 with anabolic therapy (teriparatide), in 2 with vitamin D, and in 1 with testosterone. Bisphosphonate therapy was associated, in 8 of the studies, with a significantly higher TBS change compared to the untreated controls[19-26]. However, in 2 studies this fact could not be proven, but it should be noted that in one of them there were patients with osteoporosis induced by glucocorticoids[31], and the other was carried in patients undergoing recent liver transplantation[24]. In a retrospective cohort study, broad in terms of the number of subjects, carried out by Krieg et al., changes in TBS were compared in 534 postmenopausal women treated (with compliance greater than 75%) or with bisphosphonates (86%), raloxifene (10%) or calcitonin (4%); compared to 1,150 untreated women. During the follow-up, with an average of 3.7 years, TBS reportedly increase in treated women by 0.2%/year, while it decreased in untreated women by 0.3%/year (changes that were statistically significant compared to the initial value)[28]. One of the most relevant studies that analyzes the effect of bisphosphonates on TBS was carried out by Leslie et al., in a retrospective cohort. This work is important because of the high number of subjects included (5,083 women treated, mostly with bisphosphonates -80%-, and 3,961 women without antiosteoporotic treatment) and for the long period of follow-up (average of 4.1 years). These authors found greater gains in TBS in women with greater adherence to the medication for osteoporosis (-1.2% change in TBS for untreated patients, versus +0.8% change for treated patients, with high adherence index treatment (>0.8, p for the trend <0.001) .In spite of this, and taking into account that the main objective of this study was to investigate whether the change in TBS affected the risk of fracture independently, he was able to verify this fact, concluding that the change in TBS is not a useful indicator of fracture risk[23].

Changes in TBS with bisphosphonates are generally of small magnitude. A clinical trial that evaluated the effect of zoledronic acid (at doses higher than those used in osteoporotic disease) vs. placebo in premenopausal women with breast cancer, has indicated greater increases in TBS at 2 years (of 2.41%, versus -2.16% of the placebo group)[22].

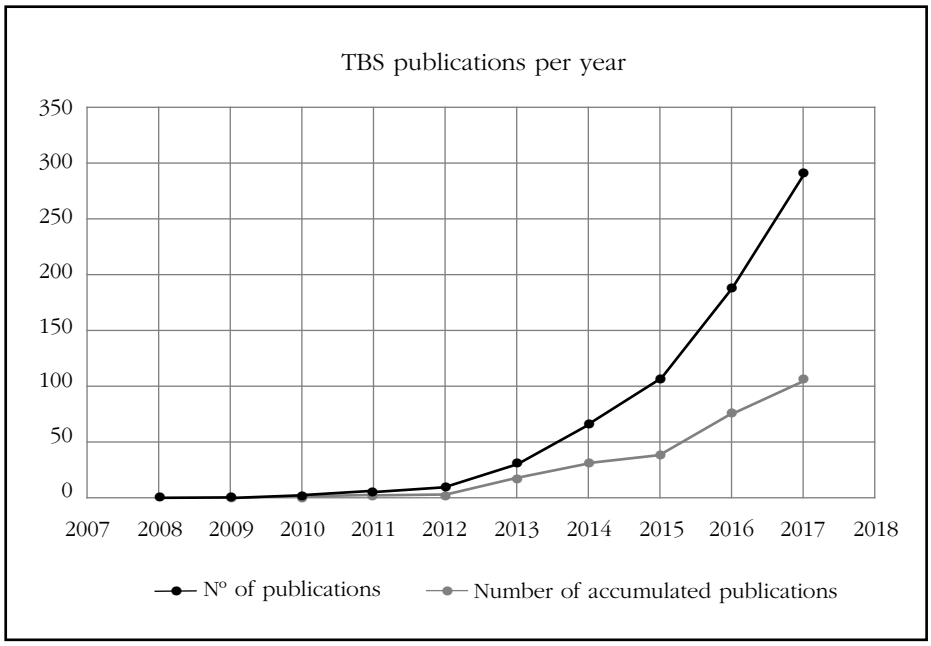
Anabolic medication was also associated with significant increases in TBS consistently in 4 studies[19,20,31,34] and in some cases this effect is described as early as 3 months after teriparatide treatment has commenced[27]. These changes are of greater magnitude than those indicated for bisphosphonates. The increase in TBS has been demonstrated, both in an open longitudinal study of patients with primary osteoporosis[13] and in a subanalysis of a clinical trial of patients with osteoporosis due to corticosteroids, in which the effect of teriparatide vs. alendronate was compared[20]. In the latter, a greater TBS increase is also shown in the group with anabolic therapy, reaching 3.6% at 36 months against the baseline value, in the teriparatide branch. In the DATA-Switch clinical trial, Tsai et al. reported that after 48 months of treatment, TBS increased by an average value of 5.1, 3.6, and 6.1% with sequential teriparatide therapy, denosumab, denosumab-teriparatide or the combination of both, respectively[33]. Similarly, although an open two-year study, Senn et al., compared changes in TBS in 65 patients treated with teriparatide vs. 122 treated with ibandronate, showed that patients treated with teriparatide had a 4.3% increase in the TBS ($p<0.001$ compared to the initial value) and significantly higher than that observed in the group treated with ibandronate (0.3%)[13]. On the other hand, only one study, with low statistical power (only 14 subjects), that assessed TBS in patients with atypical fractures and treatment with teriparatide, did not observe significant changes in this index[34].

Other research studies of denosumab antiresorptive therapy also reported significant improvement in TBS[19,29,32,33]. Recently, McClung et al. compared TBS and BMD in 157 postmenopausal women treated with denosumab versus 128 women with placebo in a subanalysis of patients in the FREEDOM clinical trial. In the denosumab group, progressive increases were seen from baseline at 12, 24 and 36 months for TBS (1.4, 1.9 and 2.4%, respectively). The percentage changes in TBS were statistically significant compared to baseline and placebo, in addition to being, to a large extent, independent of BMD and changes in BMD, induced both by time and by the effect of treatment[32]. Increases in TBS of greater magnitude have also been reported in postmenopausal women with osteopororis corticoidea after one year of treatment with denosumab, reaching an average TBS increase of 5%[26].

Interestingly, TBS changes have also been used to evaluate the effect of switching from one treatment to another. In this sense, Ebina et al.,[20] in a nonrandomized observational study, found in

women with rheumatoid arthritis and corticosteroid treatment that the change in the treatment from bisphosphonates to teriparatide produced an increase in TBS greater than the change to denosumab (2.1. vs. -0.7%). In addition, the change to teriparatide attained a significantly higher elevation in TBS than that obtained in the group that continued with bisphosphonates (2.1 vs. -1.8%)[20]. Similarly, Tsai et al. found, after 48 months of follow-up in a subanalysis of a clinical trial, that the change from teriparatide to denosumab increased the TBS with a greater magnitude than did the change from denosumab to teriparatide (5.8 vs. 3.6%, respectively)[33].

Figure 1. Number of presentations and publications on TBS presented in the period 2008-2017 (provided by courtesy of Medimaps)



Changes in TBS induced in patients by calcium and vitamin D treatment were less consistent. In a study carried out in 87 patients followed over 24 months, TBS results showed higher values compared to patients who had not received treatment[19]. However, these results were not replicated in a clinical trial that compared the effects on TBS of low dose and high dose of cholecalciferol versus placebo, after 12 months, in 230 postmenopausal women[21].

The effect of testosterone treatment on TBS has only been evaluated in a study carried out in a small group of male patients with testosterone deficiency and with substitution treatment, showing a significant increase of 5% at 24 months[19].

In most of the studies reviewed, the relationship of TBS and BMD values was reportedly low, and after treatment with the different antiosteoporotic drugs, the changes induced in BMD were clearly superior to those obtained with TBS. The relationship between both parameters is lost. This situation is especially noteworthy in the treatment with bisphosphonates.

Much of the scientific evidence reviewed shows that TBS provides a complementary and largely independent value to BMD measurements, so it is not expected that the response to bony changes by an antiosteoporotic treatment will be similar. Bone changes with TBS are especially modest in the treatment with bisphosphonates, in many cases remaining below the CMS. This has led the International Society of Clinical Densitometry (ISCD) not to recommend TBS in the monitoring of the response to the treatment of osteoporosis with bisphosphonates[35,36].

3. Question: In what diseases is TBS especially useful?

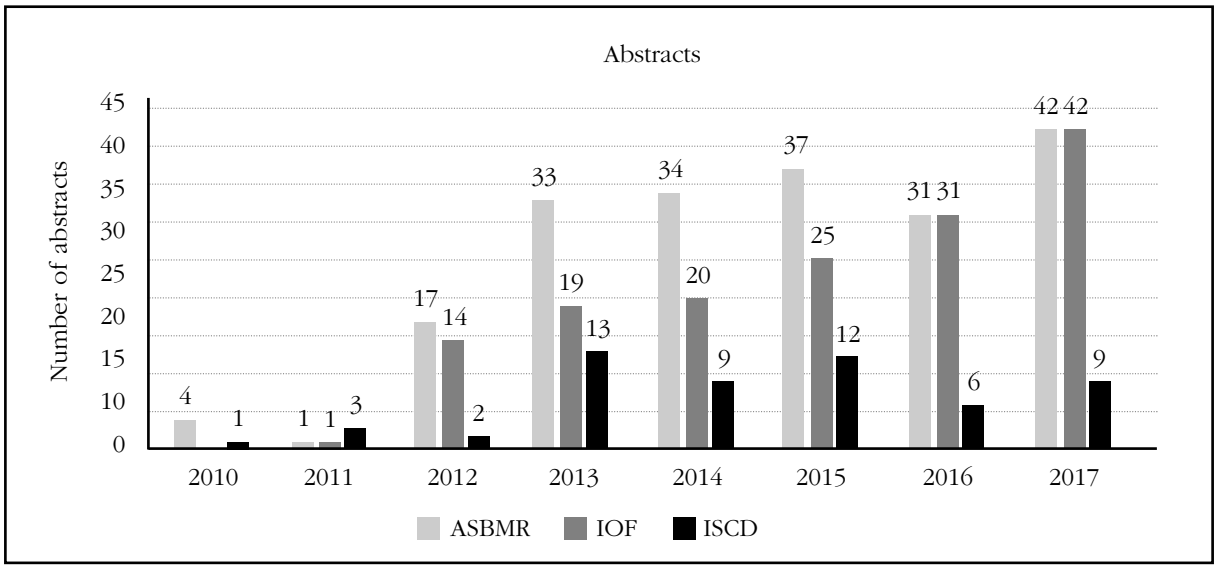
Proposal for statement 1: TBS can be used to assess the risk of fracture in subjects with diabetes.

- Evidence grade, 2+.

Summary: Patients with type 2 diabetes (DM2) have paradoxically a higher BMD and an increased risk of fragility fractures. In 8 studies it has been shown that, although BMD tends to be higher in type 2 diabetics than in non-diabetics, TBS tends to be lower in type 2 diabetics than in non-diabetics.

A cross-sectional case-control study conducted by Dhaliwal et al.[37] compared 57 women with type 2 diabetes with 43 women without it. TBS was lower and BMD increased among diabetics (p = 0.001 and 0.01, respectively). On the other hand, the TBS was lower (p=0.01) and the BMD did not show significant differences in diabetics with poor glycemic control compared to those with good glycemic control (previous A1c <7.5%). These data were confirmed in a larger study[38] that included 1,229 men and 1,529 postmenopausal women older than 50 years of the Korean Ansung cohort. TBS in the lumbar spine was significantly lower in women and men with diabetes than in non-diabetic women and men, while BMD in the lumbar spine was significantly higher in subjects with diabetes. Other recent case-control studies confirmed these findings in 131 diabetic patients and 265 controls[39] and in 88 diabetic patients and 88 controls[40]. Holloway et al.[41] observed the same trend in subjects with normoglycaemia, patients with high fasting fasting glucose (GAB) and diabetic patients. Diabetic or high GBA patients had higher BMD in the lumbar spine and lower TBS than patients with normoglycemia[41].

Figure 2. Abstracts on the TBS presented to congresses of the ASBMR, IOF, ISCD (provided by courtesy of Medimaps)



Iki et al.[42] observed a significantly higher BMD in men with diabetes compared to controls but did not observe significant differences in TBS. Fasting blood glucose levels, HbA1c and HOMA-IR (homeostasis model assessment index) correlated significantly inversely with TBS after adjusting for age, BMI and BMD. The multivariate linear regression analysis revealed that the glycemic indexes (GBA and HbA1c) were significantly associated with an increase in BMD and a decreased TBS, and that the evaluation of insulin resistance by the HOMA model was only associated with the TBS These associations were not modified after further adjustment for markers of bone turnover and pentosidine levels. These data were confirmed in a Korean population study (894 controls and 325 diabetic patients) where TBS was also negatively correlated with GBA, HbA1c and HOMA-IR.

Leslie et al.[43] included in a study 29,407 Canadian women aged 50 years or older with reference DXA scans, of which 2,356 had been diagnosed with diabetes. After adjusting for clinical risk factors, it was found that diabetic women were more likely to be in the lower tertile of lumbar TBS, but were less likely to be in the lower tertiles of BMD of the lumbar spine, neck of the femur or total femoral area. The TBS values were a predictor of incident fractures independent of BMD.

In addition, Zhukouskaya et al.[44] evaluated how the TBS and BMD variables could be useful to identify vertebral fractures (FxV) in a cohort of 99 patients (postmenopausal women) with well-compensated type 2 diabetes (T2D). They compared these patients with T2D with 107 control subjects without T2D. They found that patients with DM2 had a higher prevalence of FxV compared to controls (34.3 vs. 18.7%, p=0.01). TBS was not different between well compensated type 2 diabetic patients and controls, but interestingly, TBS was decreased in patients with DM2 and fractures.

On the other hand, Bonaccorci et al.[40] compared possible predictors of fractures in a group of 80 women with DM2 and 88 controls, and showed that TBS (AUC=0.71) and adjusted FRAX® for TBS (AUC=0,74) were the only statistically significant parameters in the diabetic group, unlike BMD and structural analysis of the femur. Finally, Choi et al.[45], in a study conducted with 169 Korean postmenopausal women with DM2, found a significantly lower TBS (p=0.008) and a FRAX® score adjusted for the highest TBS (p=0.019) in the group. with FxV compared to the group without FxV. In contrast, there were no significant differences in BMD and original FRAX® scores between the 2 groups. The TBS (OR=1.8 [95% CI: 1.1-2.7], p=0.011) and the FRAX® score adjusted by the TBS (OR=2.0 [95% CI: 1.1-3.5], p=0.020) showed statistically significant ORs for FxV. The TBS and the FRAX® adjusted by TBS could be supplementary tools to discriminate osteoporotic fractures in DM2.

Proposal for statement 2: The TBS could be useful to evaluate the risk of fracture in subjects treated with glucocorticoids or endogenous hypercortisolism.

- Evidence grade, 2+.

Summary: Glucocorticoids (GC) produce rapid bone loss and an increased risk of fracture that can not be completely explained by changes in BMD. Leslie et al.[46] investigated the clinical risk factors associated with TBS. Among 29,407 women in the Manitoba cohort with DXA scans of the lumbar spine, 1,213 had a history of recent use of GC. They found that the probability of a reduced TBS value is increased in subjects with recent GC use after adjusting for BMD (OR=1.67 [95% CI: 1.40-1.99]). On the other hand, Leib et al. and Paggiosi et al.[47,48] showed that TBS decreases in subjects treated with glucocorticoids and that TBS is more sensitive than BMD in these subjects. In their study, Paggiosi et al.[48], who evaluated 484 women (mean age 67±7.5 years) of whom 64 had

taken prednisolone (mean dose of 7.2±3.2 mg/day, mean duration of 9.2 ±10.8 years), found that subjects with GC had a significant decrease in TBS compared to women without prior treatment with GC, and there were no differences in BMD of the lumbar spine. These results were corroborated in a larger-scale study by Leib et al.[47]. This study involved 1,520 men and women aged 40 years or older. Among them, 416 subjects who received GC (dose ≥5 mg/day, for ≥3 months) were compared with 1,104 control subjects adjusted for similar sex, age and BMI. The authors demonstrated a significant decrease in TBS (p<0.001) compared to controls, whereas no change was observed in the BMD of the lumbar spine (p=0.88). In addition, they observed more pronounced decreases in TBS in males compared to females. Finally, they observed that this alteration of the TBS was even more pronounced when the subjects with GC and fracture were compared with the subjects with GC without fracture (p<0.01), or when compared with the controls (p<0.001). This study showed that TBS was associated with the presence of a fracture with an OR of 1.51 [95% CI: 1.23-1.86] per DE decrease in TBS and an AUC of 0.648 [95% CI: 0.599-0.693]. A recent small study by Chuang et al.[49] confirmed these trends in 30 patients who received GC therapy for 24 months and in 16 without it. The results showed a significant decrease in the percentage change in the TBS for the lumbar spine and a greater probability of fracture estimated by FRAX® adjusted by the TBS.

One of the endogenous forms of glucocorticoid-induced osteoporosis (OIG) is the presence of adrenal incidentaloma (IS), which can induce subclinical hypercortisolism and increase the risk of fracture. In a cohort of 102 patients[50], the authors established that subjects with SI had significantly lower TBS values than controls. It is noteworthy that patients with subclinical hypercortisolism (n=34) exhibited significantly lower TBS than those without subclinical hypercortisolism, expressed by a Z-score of TBS of -3.18±1.21 vs. -1.70±1.54 (p<0.0001), despite having a Z-score of normal BMD in the spine and femur. Finally, lumbar TBS was a predictor of incident fractures in an average of 40 months of follow-up, regardless of the patient's age, BMI and BMD of the lumbar spine. However, Belaya et al.[51] found in a population of 182 patients with subclinical hypercortisolism that only the level of free cortisol in 24 h urine (24 hUFC) was the only predictor of fracture. These authors observed low TBS values in their population (average Z-score of the TBS= -1.86), while the decrease in BMD was lower than the average Z-score of the BMD= -1,60).

Proposal for statement 3: TBS may be useful in the clinical evaluation of patients with primary hyperparathyroidism.

- Evidence grade, 2+.

Summary: In primary hyperparathyroidism (PHPT), vertebral fractures (FxV) occur independently of BMD and may depend on the decrease in bone quality.

In their cross-sectional study, Romagnoli et al.[12] observed a significantly lower TBS in 73 postmenopausal women with primary hyperparathyroidism (29 of them with a documented vertebral fracture) than in 74 controls of similar age. In addition, the presence of vertebral fractures was associated independently with the reduction of TBS (OR=0.003 [95% CI: 0-0.534], p=0.028). In a study that included both transverse and longitudinal components, Eller-Vainicher et al.[52] compared 92 patients with primary hyperparathyroidism (74 of them were postmenopausal women and 18 were men older than 50 years) with the results of 98 controls recruited simultaneously in the clinic. In agreement with the previous study, TBS was lower in patients with primary hyperparathyroidism than in controls, and was significantly associated with vertebral fracture, even after adjustment for age, sex, BMI and BMD of the lumbar spine (adjusted OR=1,4 [95% CI: 1.1-1.9]). In the longitudinal phase of the study, 20 patients with primary hyperparathyroidism who underwent an effective parathyroidectomy were compared at 24 months of follow-up with 10 patients treated conservatively. In the surgery group, the average TBS score increased by 47% (p<0.01). In patients followed conservatively, TBS decreased significantly compared to non-fractured patients (p<0.048).

Finally, Silva et al.[53] evaluated the relationship between TBS, high resolution peripheral quantitative computed tomography (HR-pQCT) and bone resistance (by finite element analysis) in distal radius and tibia in 22 postmenopausal women with mild primary hyperparathyroidism. They found that TBS was correlated with complete bone strength and all HR-pQCT indices, except for trabecular thickness and trabecular stiffness in the radius, whereas TBS was correlated with volumetric densities, cortical thickness, trabecular bone volume and the complete bone resistance of the tibia. The conclusion was that the TBS is a promising diagnostic tool in the clinical evaluation of the trabecular microstructure in those patients who suffer a milder form of primary hyperparathyroidism.

In patients with asymptomatic PHPT, Diaz-Soto et al.[54] did not find significant differences in the TBS when comparing normocalcemic vs. hypercalcemic patients. Cipriani et al.[55] investigated skeletal changes after the restoration of the euparatiroid state, and, unlike Rolighed et al.[56], found no significant changes in TBS after parathyroidectomy in patients with PHPT. However, they found a significant increase in TBS after 18 months of treatment with recombinant parathormone (rhPTH) in hypoparathyroid patients.

Proposal for statement 4: TBS could be useful to assess bone fragility in patients with severe osteoarthritis.

- Evidence grade, 2+.

Summary: Lumbar osteoarthritis overestimates bone density measured by DXA.

In these studies, the impact of osteoarthritis of the lumbar spine on the TBS result was assessed based on a French cohort of 390 women aged 50

or older[11] and a part of the OPUS cohort that included 727 postmenopausal women of 55 years of age or more[57]. In the study by Dufour et al.[11], the presence of osteoarthritis was evaluated using the ISCD definition (a difference of more than 1 SD in the T-score between two adjacent vertebrae). In the study by Kolta et al.[57], they used the Kellgren and Lawrence (KL) classification based on radiographs of the lateral lumbar spine. In both studies significant differences were observed between those with and without osteoarthritis in the bone mineral density measured by DXA. In the study by Kolta et al., The increase in BMD correlated with the severity of osteoarthritis (KL scale). However, the TBS values were not influenced by the presence of osteoarthritis in both studies[11,57].

Review of the scientific evidence on the clinical use of TBS: Official positions of the SEIOMM

Summary

1. Question: Can TBS be used to assess the risk of fracture in clinical practice?

- TBS can be used to assess the risk of vertebral fracture, femur and global fragility in women and men from 50 years of age.
  [Level of evidence, 2 ++. Degree of recommendation, B]
- TBS can be used in conjunction with BMD to assess vertebral, femur and global fragility in men and women from 50 years of age.
  [Level of evidence, 2 ++. Degree of recommendation, B]

2. Question: Can TBS be used to monitor patients with osteoporosis?

- The TBS can be used to evaluate changes over time.
  [Level of evidence, 2+. Degree of recommendation, C]
- TBS does not improve BMD in the assessment of the effect of treatment over time. It should not be used in the assessment of response to bisphosphonates.
  [Evidence level, 2 ++. Degree of recommendation, B]

3. Question: In what diseases is TBS especially useful?

- TBS can be used to assess the risk of fracture in subjects with diabetes.
- TBS can be used to assess the risk of fracture in subjects treated with glucocorticoids.
- TBS can be used for the clinical orientation of subjects suffering from hypo and hyperparathyroidism.
- TBS can be used for the diagnostic orientation of patients in the presence of osteoarthritis.
  [Level of evidence, 2+]

Table 2. Levels of evidence and degrees of recommendation of the Scottish Intercollegiate Guidelines Network (SIGN)

Levels of scientific evidence	
1++	High quality meta-analysis, systematic reviews of clinical trials or high-quality clinical trials with very little risk of bias
1+	Well conducted meta-analyzes, systematic reviews of clinical trials or well-conducted clinical trials with little risk of bias
1-	Meta-analyzes, systematic reviews of clinical trials or clinical trials with a high risk of bias
2++	High quality systematic reviews of cohort or case-control studies. Cohort or case-control studies with very low risk of bias and with a high probability of establishing a causal relationship
2+	Cohort studies or well-conducted cases and controls with low risk of bias and with a moderate probability of establishing a causal relationship
2-	Cohort or case-control studies with high risk of bias and significant risk that the relationship is not causal
3	Non-analytical studies, such as case reports and case series
4	Expert opinion
Degrees of recommendation	
A	At least one meta-analysis, systematic review or clinical trial classified as 1++ and directly applicable to the target population of the guideline; or a volume of scientific evidence composed of studies classified as 1+ and with great consistency among them
B	A volume of scientific evidence composed of studies classified as 2++, directly applicable to the target population of the guide and showing great consistency between them; or scientific evidence extrapolated from studies classified as 1++ or 1+
C	A volume of scientific evidence composed of studies classified as 2+ directly applicable to the target population of the guide and showing great consistency among them; or scientific evidence extrapolated from studies classified as 2++
D	Scientific evidence of level 3 or 4; or scientific evidence extrapolated from studies classified as 2+

Studies classified as 1- and 2- should not be used in the process of making recommendations because of their high potential for bias.

Good practice guideline

√[1] Recommended practice, based on the clinical experience and the consensus of the writing team

1. Sometimes the development group realizes that there is some important practical aspect on which they want to emphasize and for which there is probably no scientific evidence to support it. In general, these cases are related to some aspect of the treatment considered good clinical practice and that nobody would generally question. These aspects are valued as points of good clinical practice. These messages are not an alternative to recommendations based on scientific evidence, but should be considered only when there is no other way to highlight this aspect.

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