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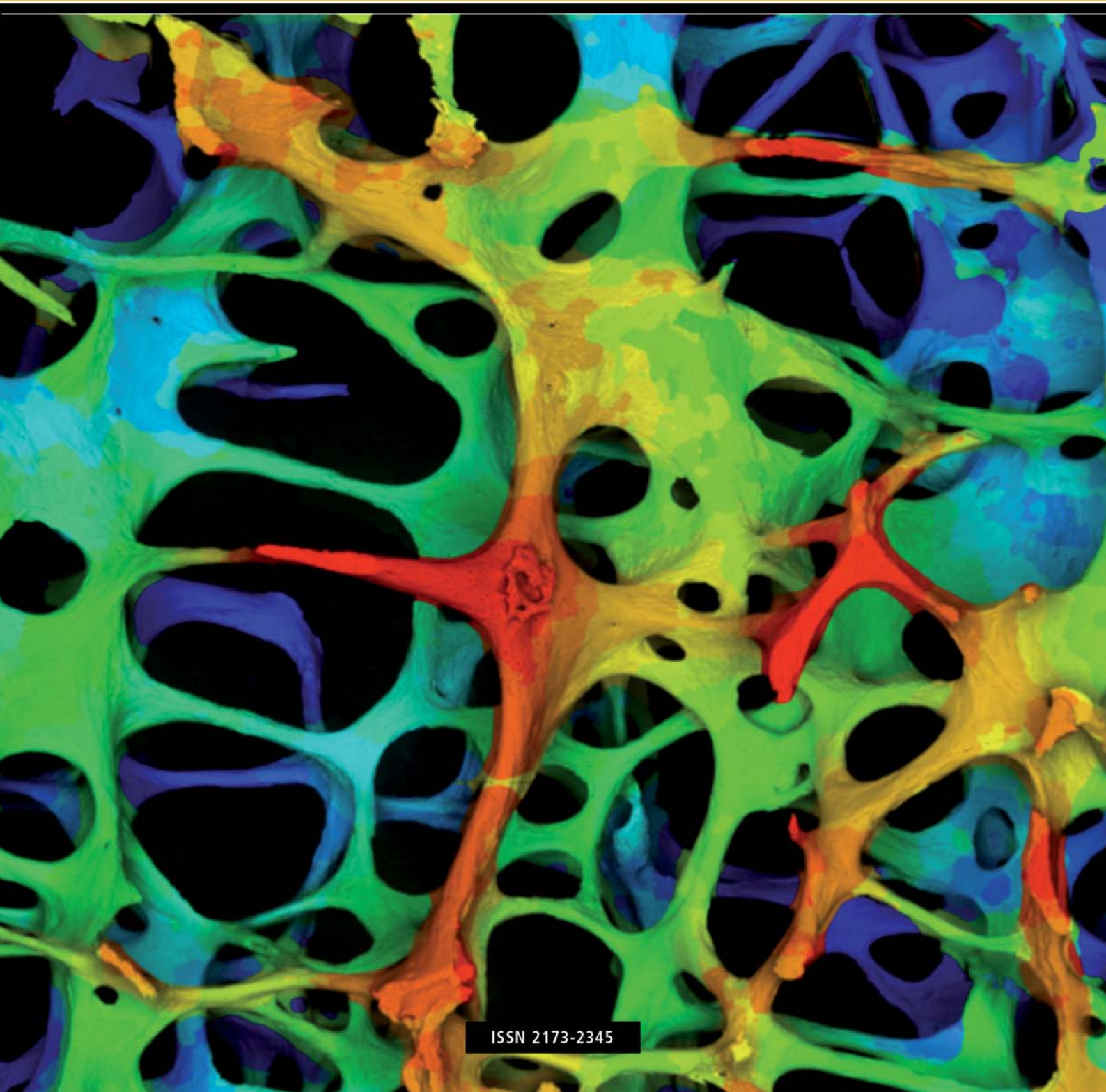
Volume 8 · Number 1 · January-March 2016

Revista de Osteoporosis y Metabolismo Mineral

www.revistadeosteoporosisymetabolismomineral.com



ISSN 2173-2345

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XXI Congreso de la SEIOMM - 19, 20 y 21 de Octubre de 2016

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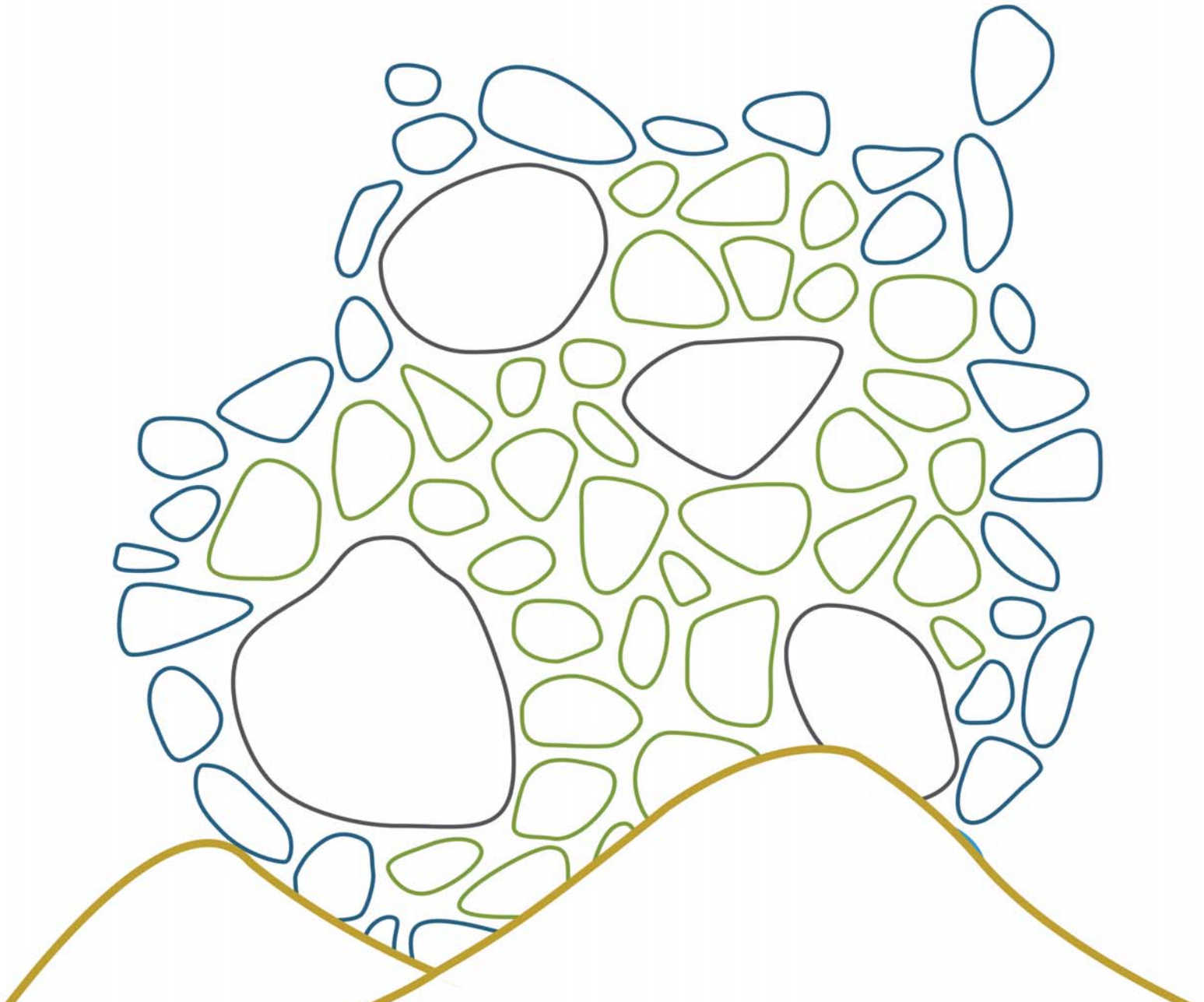

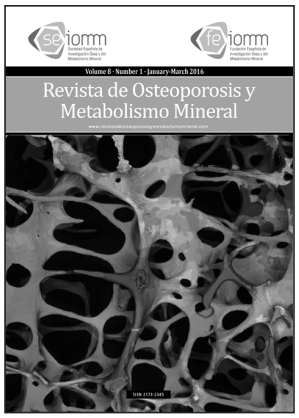

Our cover
Trabecular bone

Autor:
Courtesy of Professor Alan Boyde. London. United Kingdom

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ISSN: **2173-2345**

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SUMMARY

Vol. 8 - Nº 1 - January-March 2016

Indexed in: Scielo, IBECS, SIIC Data Bases, embase, Redalyc, Open J-Gate, DOAJ, Free Medical Journal, Google Academic, Medes, Electronic Journals Library AZB, e-revistas, WorldCat, Latindex, EBSCOhost, MedicLatina, Dialnet, SafetyLit, Mosby's, Encare, Academic Keys.

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Proper compliance of treatment for osteoporosis: we still have much to do

DOI: http://dx.doi.org/10.4321/S1889-836X2016000100001

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Osteoporosis is a common disease, its main clinical complication being bone fragility[1]. This chronic, generally assymptomatic process deteriorates the bone, exposing it to fracture risk. Current treatment techniques aim to minimize the possibility of new fractures[1-4] but there is no medication to eliminate such risk. Most drugs currently available for treating osteoporosis achieve reductions of between 40 and 65%[2-4], if the medication is taken continuously over a period ranging from 3 to 5 years. This would be mere utopian, as, in fact, the patients frequently abandon their osteoporosis treatment, once they have begun.

Numerous studies have shown that adherence to osteoporosis treatment is generally low, and that in the first year the dropout rate is between 30-50% in most cases[5]. One reason may be their asymptomatic condition, which does not provide the patient with a sense of improvement. Perhaps, if all goes well, the patient does not suffer fracture, but subjectively does not perceive anything. In this respect, osteoporosis differs from other chronic diseases in which symptoms return as soon as the patient discontinues treatment, such as migraines, ischemic heart disease or diabetes mellitus.

The treatment procedure could be another factor related to the patient's carrying on correctly with their prescribed medication. Several studies have shown that compliance with bisphosphonate was better when the doses were spaced. Thus, Penning-van Beest et al observed that after one year, 51.9% of patients continued treatment with weekly administration, whereas only 30-42% of those with daily doses, regardless of the type of bisphosphonate administered (etidronate, alendronate or risedronate)[6]. In another study, Cramer et al assessed the compliance of 2,741 women treated with bisphosphonates and found that, after one year, adherence was 44.2% in those taking it on a weekly basis, compared to 31.7% among those on a daily regime[7].

In the US, Ettinger et al analyzed sales of alendronate and risedronate prescriptions in more than 211,000 women and found that after one year, 56.7% of patients taking weekly bisphosphonate continued taking the drug, compared with 39% of those who took the medication daily. These authors pointed out, however, that over 40% of patients continued weekly treatment with bisphosphonates[8]. In a study of 15,640 women in the UK, France and US, Cramer et al found that after one year, patients' adherence with bisphosphonates was higher in those receiving medication weekly, compared to daily (44% vs 32%, respectively, in the United States; 52% vs 40% in the UK, and 51% vs 44% in France), where in all cases the value of $p<0.001$[9].

There have been other studies comparing monthly and weekly administration of bisphosphonates. In the PERSIST study, adherence to treatment was compared for six months in a group of women receiving monthly ibandronate versus another taking alendronate weekly. It was found that of those taking medication monthly 56.6% kept up the treatment, compared to 38.6% of those taking alendronate weekly[10].

The introduction of zoledronate and denosumab, drugs with a longer half-life that allows an annual and biannual administration respectively, has significantly changed the scenario of therapeutic failure and patient preferences. A multi-center, randomized, double-blind study conducted by McClung et al. to assess the safety and efficacy of a single intravenous dose of 5 mg zoledronic acid vs 70 mg weekly oral alendronate, and performed in 225 women with postmenopausal osteoporosis who had previously been treated with weekly alendronate result showed that 78.7% of patients expressed a preference for intravenous versus oral treatment on a weekly basis[11], as most participants expressed in a similar study by Saag et al.[12].

Clearly, patients regularly taking medication for osteoporosis have better results, both in terms of changes in bone mineral density[13] and, more importantly, the reduced rate of fracture and lower mortality[14,15]. A study by Siris et al in a large population of postmenopausal women over 45 years, treated with bisphosphonate for osteoporosis, showed that after two years of follow-up, those women taking the treatment properly (43%) reduced the risk of fracture, both vertebral and non-vertebral, 21% higher than patients who did not follow the treatment correctly[16]. Previously, Caro et al had obtained similar results, finding a reduction in the appearance of new superior fractures (16%) among those patients who complied

compared to those who did not. In this follow-up study period of 2 years, and the treatments evaluated were calcitonin, hormone replacement therapy and bisphosphonates[17]. The same authors repeated the study using a broader base of data, with a cohort of more than 38,000 women suffering from osteoporosis, and obtained similar figures: poor adherence to treatment was associated with an increased risk of fracture of 17% after 1.7 years[18]. These results are corroborated by those obtained in other studies[19-21].

In this issue Blanch et al complete the perspective of non-compliance and poor adherence to treatment of osteoporosis from the physicians' point of view[22]. So far, most studies have analyzed the views of patients, that the degree of abandonment existed, the reasons and consequences, but the doctors' opinion regarding this matter had been rarely considered and is now published here. The authors interviewed 235 doctors throughout Spain and among other findings obtained responses that, so far, had not been collected in other studies, which gives it an additional value for its originality. Thus, among the reasons that cause nonadherence, lack of coordination between levels of care, polypharmacy and side effects are suggested.

Understanding these facts can help us gain better adherence and compliance by patients, in addition to improved levels of communication between doctors and patients. Knowledge of the expected side effects (frequency) an, when indicated, the introduction of drugs with a longer life and a semiannual or annual administration, could be useful.

Conflict of interests: The authors declare no conflict of interest in relation to this work.

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Study of miRNAs expression patterns in osteoporotic bone

DOI: http://dx.doi.org/10.4321/S1889-836X2016000100002

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Date of receipt: 20/10/2015
Date of acceptance: 10/12/2015

Work scholarship from the SEIOMM to attend the 36th Congress of the ASBMR (Houston, 2014).

Summary

Objectives: To identify microRNAs (miRNAs) differentially expressed in bone samples with osteoporotic fracture compared with healthy bones.

Methods: Total RNA was extracted from fresh trabecular bone of the femoral neck of women undergoing hip replacement surgery, either because to osteoporotic fracture (n=6) or in the absence of osteoarthritis osteoporosis (based on BMD) (n=6). The samples were hybridized on an array of miRNAs and PCA diagrams and heat map were made. To compare expression levels, >1.5 times and a value $p<0.05$ Student's T test (corrected for multiple testing) was set as a threshold of significant change.

Results: Both PCA analysis and the heat map showed a samples grouping whether there was fracture or not. 790 were detected miRNAs in bone samples, 82 of which were altered in the osteoporotic samples. After validation in another panel of 6 samples 6 osteoporotic and non-osteoporotic by PCR real time of the most significant miRNAs, and for which there was a test available, the miRNAs, miR-320a and miR-22-3p were confirmed. These two miRNAs were detected in cultures of primary osteoblasts, although they did not maintain the same pattern of expression in total bone samples.

Conclusions: We have shown that there are differences in the expression of miRNAs in samples with osteoporotic fracture. This opens prospects for research and design of new therapies.

Key words: *microRNA, bone fracture, osteoporosis, osteoblasts, bone.*

Introduction

MicroRNAs (miRNAs) have been linked to a variety of processes, such as cellular proliferation, differentiation and apoptosis. Deregulation of any of these processes could lead to pathological disorders, some as severe as cancer[1].

MicroRNAs are small (18-24nt), non-coded RNAs, that negatively regulate gene expression by binding to the 3'-UTRs of the target miRNAs sequences. In bone tissue, miRNAs have been described as key factors in regulating the formation, remodeling and homeostasis of the bone[2,3]. Furthermore, several studies have shown that miRNAs are involved in controlling the differentiation and function of bone cells[4].

Thus the identification of such miRNAs could be a tool to develop therapies to promote bone formation or inhibit bone resorption and so act on bone diseases.

In the field of osteoporosis there are very few studies concerning miRNA involvement in its pathophysiology. Li et al.[5] described a mutation in the pre-miR-2861 that blocked miR-2861expression, causing primary osteoporosis in two adolescent relations. In another study, three polymorphisms in the target sequences for miR-146a and miR-146b in the FGF2 gene is genetically associated with bone mineral density (BMD) of the femoral neck[6]. There have also been several studies which attempted to identify miRNAs with an altered expression pattern associated with osteoporotic fracture to find disease biomarkers[7-9]. All of these studies found different miRNAs proving once again the complexity of osteoporosis fracture.

However, the sample type, the conditions of collection and handling process as well as the characteristics of the study population may influence the final result. In fact, a variety of expression profiles of miRNAs have been found depending on the cell type studied within the osteoblast lineage (proliferation, differentiation, mineralization), if subjected to any treatment (hormones, cytokines) or species of origin[10,11]. Thus, it is difficult to compare studies, although any input in the field is a step closer to understanding the pathophysiology of the fracture.

The aim of this study was to identify miRNAs with altered expression in osteoporotic bone, using an experimental methodology as close as possible to physiological conditions. For this purpose, the trabecular bone obtained from patients with a recent osteoporotic fracture compared to non-osteoporotic bone samples were analyzed. A study of microarray hybridization was performed using fresh whole bone tissue to detect all miRNAs expressed in these samples.

Material and methods

Preparation of the bone samples

The trabecular bone of the femoral neck was obtained from postmenopausal women undergoing hip replacement, either osteoporotic fracture (OP) (n=6) or osteoarthritis (n=6). Samples in the absence of osteoarthritic osteoporosis were determined by measuring BMD and considered as the control group. To validate the results of the array, six additional samples of the trabecular bone of the femoral neck with fracture were obtained and 6 samples without osteoporosis. Of the latter samples, primary osteoblasts (HOB) were also obtained. No patient presented a history of metabolic or endocrine disease, chronic renal failure, chronic liver disease, cancer, Paget's disease, malabsorption syndrome, treatment of hormone replacement therapy, or medication agents or oral anabolic steroids, anti-epileptic drugs, lithium heparin or warfarin. Written informed consent was obtained in accordance with the regulations of the Ethics Committee of the MAR Health Park Clinical Research Center, which approved the study.

Cultivation of primary osteoblasts

For the cultivation of osteoblasts from the bone samples of the validation phase, small fragments of trabecular bone were obtained and placed in culture plates of 140 mm. They were incubated with DMEM (Dulbecco's Modified Eagle Medium) culture medium supplemented with FBS (Fetal Bovine Serum) 10% penicillin/streptomycin 1%, 0.4% fungizone and 100 μg/ml ascorbic acid. The plates were trypsinized after about three weeks just before the junction for RNA extraction.

RNA extraction

For RNA extraction, total bone tissue, fresh samples of trabecular bone were cut into small pieces, washed three times in phosphate buffered saline (PBS), and stored at -80 until use.

The RNA extraction of both total bone and HOB was performed using miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions. For primary osteoblasts, the RNeasy MinElute Cleanup (Qiagen) was also used to obtain the fraction enriched miRNAs. The concentration of purified RNA was analyzed in a spectrophotometer (Nanodrop, Thermo Fisher Scientific Inc).

Microarray microRNAs of total bone samples and data analysis

The microarray and data analysis were performed on the Exiqon (Denmark) platform platform. The quality of total RNA was verified by the Agilent 2100 Bioanalyzer and 250 ng of RNA, both as reference samples, were labeled with fluorescent signals HY3™ and HY5™, respectively, using the miRCURY LNA™ microRNA Hi-Power Labeling Kit, HY3™/HY5™ (Exiqon, Denmark) following the procedure described by the manufacturer. The RNA samples, labeled HY3™, and the sample labeled reference HY5™ were mixed equally and hybridized to the array of miRCURY LNA™ microRNA (Exiqon, Denmark) containing capture probes for all human, mouse or rat miRNAs, entered into the miRBase 18.0. Hybridization was carried out

following the array instructions using a Tecan HS4800 hybridization station™ (Tecan, Austria). After hybridization, the results were scanned and stored in an environment free of ozone (<2.0 ppb ozone) to prevent the extinction of the fluorescent markers. Scanning was carried out using the Agilent Microarray Scanner System G2565BA (Agilent Technologies, Inc., USA). Image analysis was performed using ImaGene® 9 (Software Analysis miRCURY LNA™ microRNA, Exiqon, Denmark). Signals were quantified (Normexp With offset value 10, see Ritchie et al.[12]) and normalized using the global regression algorithm Lowess (locally weighted scatterplot smoothing). After normalization, unsupervised and supervised data analysis took place. Diagrams of principal component analysis (PCA) and heat map showing performed unsupervised hierarchical clustering. The expression levels were compared using the Student t test. The significance threshold was established in the change log (logC) >1.5 times and a value of $p<0.05$.

Validation of miRNAs differentially expressed between the OP group and the control group

The quantification of the expression of miRNAs in samples of whole blood was performed by real-time PCR (qPCR) on the Exiqon (Denmark) platform. 10 ng of RNA were transcribed to cDNA using the kit miRCURY LNA™ Universal RT microRNA PCR, polyadenylation and cDNA synthesis according to the instructions. The cDNA was diluted 100-fold and the expression of each of the miRNAs was quantified by qPCR using a custom panel of specific primers (panel custom pick & mix, Exiqon) and ExiLENT SYBR® Green mastermix. Negative controls were run in parallel with the samples. Amplification was performed on a LightCycler 480 Real-Time PCR System (Roche) in 384-well plates. Amplification curves were analyzed using the software Roche LC Cq for determining the value (the second derivative method). Differences in expression levels between the two groups was calculated as 2^ddCq. Amplification efficiency was calculated using algorithms similar to LinReg software. In the analysis of the test data, only 3 Ct detected below the negative control and Ct <37 were included. Normalization was performed based on the test average detected in all samples, as it is proven to be the best method for normalization of qPCR studies involving numerous tests[13]. For the present study, this included 11 trials. The stability of the mean of 11 miRNAs was higher than any miRNA alone in the measured data set by NormFinder software[14].

The formula used to calculate normalized values Cq (DCQ) is:

Standard Cq = Cq Media - Cq trial (sample)

Finally, the quality control of the data was performed as well as unsupervised data analysis, Student t and Wilcoxon test for comparison between groups ($p<0.05$ was considered significant).

Validation primary osteoblasts

To prepare cDNA, 1 µg of RNA was retrotranscribed from each sample using the miScript II RT kit (Qiagen).

The expression of miRNAs was quantified by qPCR using the miScript SYBR Green PCR Kit using the sequence of the mature miRNA as the first. The cDNA was diluted at a ratio of 1/5 and 0.5 µl of sample per well was used, following the protocol described by the supplier. Amplification was performed in the "Flex QuantStudio 12K Real-Time PCR" system in 384-well plates, and the data were analyzed with "expression suite" software. Expression was analyzed by RQ relative quantification using the method of the second derivative (DDCt). The U6 snRNA was used as endogenous control to normalize the samples. Each experiment was done in triplicate. To compare the statistical differences between the groups with and without osteoporosis, Mann-Whitney nonparametric statistical test was carried out using SPSS version 12.0 for Windows.

Bioinformatic analysis of miRNAs validated

For studying target genes differentially expressed miRNAs was used following programs: PicTar (http://pictar.mdc-berlin.de) TargetScan Human (http://www.targetscan.org) miRDB (http://mirdb org), Miranda (http://www microrna.org) DIANA-TarBase (http:.//diana.imis athena-innovation.gr) and miRTarBase (http://mirtarbase.mbc. nctu.edu.tw). TARGET-mirPath computer based tool web (11) to identify potentially altered molecular pathways and the intersection of the miRNAs differentially expressed in the fractured bone was used. Information about protein function was obtained in the UniProtKB database (http:// www.uniprot.org).

Results

Description of the study patients

Anthropometric characteristics, both of patients whose bone samples were used for expression array, as well as those used to perform validations array results are shown in Table 1. There were no differences in age and body mass index between the two groups of patients (Mann-Whitney test).

Unsupervised analysis of the expression array

Each trabecular bone sample of all the patients in the study bone was analyzed individually in the microarray miRNAs, and unsupervised analysis of the results was carried out based on the expression profile in order to identify variation patterns related to biological and technical factors. Principal component analysis (PCA) was performed which included 50 miRNAs with greater expression variation between samples to get an overview of the clustering of the samples according to their variance (Figure 1). The group of non-osteoporotic samples (control group) showed a much more homogeneous profile than the osteoporotic samples. The O-500 sample obtai-

ned from a patient with osteoporosis, was considered atypical and excluded from the analysis. The heat map diagram corroborates the PCA results with a clear grouping of the control samples and a sparse array of osteoporotic samples (Figure 2).

Comparison of expression of microRNAs between OP and controls in total bone samples

The mean expression levels of miRNAs were compared between groups with and without osteoporosis, excluding the O-500 samples. This analysis identified a subset of 82 miRNAs (miRNAs analyzed about 1,932) whose absolute value of the logarithm of the ratio was greater than 1.5 and with an adjusted p value less than 0.05. Seven of these miRNAs corresponded to small nucleolar RNAs, C/D box (SNORD), three to virus and one was a miRPlus™ property Exiqon sequence is not recorded in the miRBase data, and were excluded from the validation phase.

Of those remaining (Table 2), 15 hsa-miRNAs were chosen with the best significance values and for which Exiqon available probes for validation by qPCR: let-7a-5p, miR-126-5p, miR-30c- 1-3p, miR-22-3p, miR-25-3p, miR-26b-5p, miR-339-5p, miR-423-3p, miR-320a, miR-483-5p, miR-491-3p, miR-574-5p, miR-631, miR-99a-5p and miR-99b-5p. The PCA diagram of individual qPCR results showed clustering of the sample results to be very similar to the array expression, confirming the different biological source of the two sample groups (Figure 3). An osteoporotic sample (O-567) was located in the control group and was excluded from the analysis. After statistical analysis four miRNAs; miR-320a, miR-99a-5p, miR-339-5p and miR-22-3p showed significant differences between groups with osteoporosis and control (Table 3). However, the miRNAs miR-99a-5p and miR-339-5p were found to be overexpressed in OP samples results array expression, while they were under-expressed in the OP samples validation phase. These contradictory results suggest a role for these miRNAs unrelated to osteoporotic disease and they were discarded for in silico study.

Comparison of expression of microRNAs between OP and controls crops Hob

For each of the whole blood samples, primary osteoblasts were obtained. These were analyzed by qPCR expression of the miRNAs previously validated in bone tissue samples. MiRNAs miR-99a-5p and miR-339-5p were not detected in osteoblastic cells, while the miRNAs miR-320a and miR-22-3p were expressed in the HObs, although neither showed significant differences between the two biological groups.

Prediction of target genes and analysis of signaling pathways validated miRNAs

A comprehensive computer analysis by six different programs allowed us to predict potential target genes for the validated miRNAs. MiR-320a can regulate genes involved in cell proliferation (KRAS, PDGFD), inhibition of apoptosis (MCL1), signal transduction (MAPK1, SOS2, PTEN), regulation of gene expression (RUNX2, PPARGC1A, SP1, CAMTA1, ESRRG) receptors, growth factors, hormones and cytokines (NPR1, BMPR1A, AR, IGF1R, ESR1), etc. Moreover, as validated targets are the CTNNB1, TFRC and POLR3D genes. This miRNA is involved in 44 of the Encyclopedia of Genes and Genomes Kyoto (KEGG) tracks, according to predictions of the DIANA-mirPath program where the prostate cancer path is the most significant (1,105e-12), followed by endometrial cancer (2,261e-10), the mTOR signaling pathway (7,132e-08), and the PI3K-Akt signaling pathway (3,914e-07).

The miR-22-3p has many validated target genes, some of them very important in the regulation of bone metabolism. Among them are the ESR1, the PRKACA (required for adipogenic differentiation and inhibition of osteoblast differentiation), HDAC4, SP1, BMP7 and CDK6 (BMP2 signaling antagonist). The main signaling pathway KEGG This miRNA is endocytosis (4,21e-05) but also acts in important signaling pathways such as p53 (p=0.003) and MAPK (p=0.003).

Diana software tools using the validated genes (according to the Tarbase data base) shows the endocytosis pathway as the route of intersection of the two miRNAs as they share the TFRC target gene (transferrin receptor). Furthermore, if the intersection is evaluated considering the predicted target genes according to the MicroT_CDS data base, the signaling pathways are the most significant for prostate cancer and mTOR where the two miRNAs share AKT3, PTEN and IGFR1 target genes.

Discussion

This study focuses on identifying miRNAs with altered expression in osteoporotic bone. To achieve this, fresh trabecular bone was collected from patients with or without osteoporotic fracture, with homogeneous anthropometric parameters such as age, BMI and gender. We excluded patients with disorders that affect bone remodeling. Samples from osteoporotic fracture showed a clear involvement in the expression pattern of miRNAs, demonstrating that epigenetic regulation is altered in pathological bone. These results may provide a better understanding of bone biology into those who suffer an osteoporotic fracture. Also, they help identify molecules that can be used as therapeutic targets.

Unsupervised analysis of the results of miRNAs array expression from total bone samples showed that non-osteoporotic samples (control) were grouped generating a biological cluster. Furthermore, osteoporotic bone clearly differed from the control samples, showing a more dispersed distribution, suggesting that the pathophysiology of osteoporotic disease and, ultimately, bone fracture, have a heterogeneous etiology. Working

Table 1. Characteristics of patients

	n	Age (mean ± SD)	BMI (kg/m²) (mean ± SD)	BMD (g/cm²) (mean ± SD)
Array samples				
Osteoporotic fracture (OP)	6	75.2±3.5	24.4±2.8	Fracture
Control	6	72.5±7.4	26.1±3.2	0.794±0.074
Validation samples				
Osteoporotic fracture (OP)	6	76.3±7.1	27.9±2.6	Fracture
Control	6	73±6.6	27.7±3	0.882±0.158

SD: standard deviation; BMI: body mass index; BMD: bone mineral density.

with fresh human samples generates greater variability to explore established cell lines or animal models, making it difficult to see significant differences between groups. Still, this is closer to the pathophysiological situation scenario. In this regard, our study is based on samples of human bones obtained with minimal laboratory handling, which allows us to get a similar situation to *in vivo* conditions.

In the analysis of differential expression between osteoporotic and control samples, 82 miRNAs that reached significance levels with an absolute value of the logarithm of the ratio greater than 1.5 times found. Of these, 15 were tested by qPCR in a new set of samples and the miRNAs miR-99a-5p, miR-339-5p, miR-320a and miR-22-3p were finally validated. These miRNAs were evaluated in primary osteoblasts (HOB) in culture, cells from osteoporotic comparing samples versus cells from non-osteoporotic samples. MiRNAs miR-99a-5p and miR-339-5p were not detected in the HOB, suggesting another cell type as the source of these two miRNAs. Moreover, these miRNAs were found overexpressed in array osteoporotic samples whereas they were under-expressed in the validation phase. This would seem to discard these miRNAs' link to osteoporosis. MiRNAs, miR-320a and miR-22-3p expressed in the Hob, although no differences in expression levels between sample groups were observed. This could be due to the artificial conditions of the *in vitro* cell cultures that affect the expression of microRNAs, especially those involved in the regulation of important cellular functions such as proliferation and differentiation.

This shows us once again that, although the use of cell cultures, both in established and primary lines, can help researchers understand certain cellular processes. The results may differ from the actual conditions that would occur in the original tissue within the physiological environment.

Furthermore, the use of fresh tissue not manipulated for closer physiological conditions can also provide information such dissimilar results as demonstrated in studies by Seeliger et al.[7] and Garmilla-Ezquerra et al.[8]. These studies also conducted a microarray expression from whole bone samples comparing osteoporotic versus non osteoporotic samples very similar to ours, but with different findings. Note that these two earlier studies have several features that can explain these discrepancies. These include the variety of commercial arrays used and the sample size. Furthermore, these biological studies comparing groups with anthropometric characteristics or inhomogeneous clinical features, such as age, gender, body mass index and endocrine disorders (eg. diabetes mellitus), essential for the regulation of bone metabolism. Therefore, the miRNAs identified in these studies could also be linked to other external parameters, whereas in our study, the absence of concomitant diseases, and similar features between the two groups, allows us to approach in a more reliable way what happens in osteoporotic fracture.

Therefore, a major effort in our work is extremely careful control of potential confusing characteristics between cases and controls for age, sex, body mass index and metabolic diseases associated with aging. These strict inclusion criteria considerably restrict our sample size compared to similar works, where the samples are from patients with heterogeneous characteristics. Another limitation of our study is that non-osteoporotic samples used as controls from patients with osteoarthritis and, therefore, cannot rule out other bone abnormalities. For ethical reasons collecting bone it is not allowed from healthy individuals. However, in an attempt to minimize this potential problem, the sample was obtained from bone located as far as possible the arthritic lesion.

MiR-320a is conserved in human, mouse, rat and cow. This miRNA sequence is located within the basal promoter POLR3D gene, which in turn is one of their target genes and their expresión[15] silencing. Furthermore, it has been observed that miR-320a is involved in regulating osteoblast function as it has as target the CTNNB1 gene (encoding B-catenin)[16] and RUNX2[17].

Figure 1. (A) PCA graph and (B) Diagram of the matrix of PCA. Principal component analysis with the 50 microRNAs with higher standard deviation was performed. The O-500 sample was removed from the study and was not taken into account for further analysis

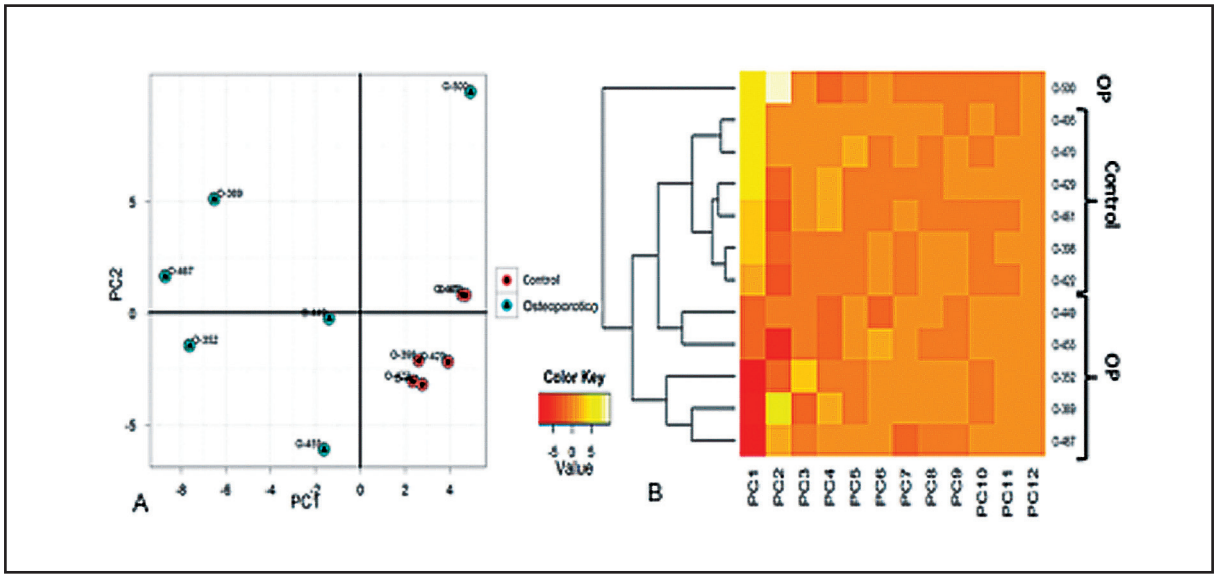

Figure 2. Diagram of heat map: The diagram shows the result of hierarchical clustering of microRNAs and samples. Each row represents a microRNA and each column, a sample. The color scale illustrates the relative expression level of microRNAs: red, below the reference channel; green, higher than the reference

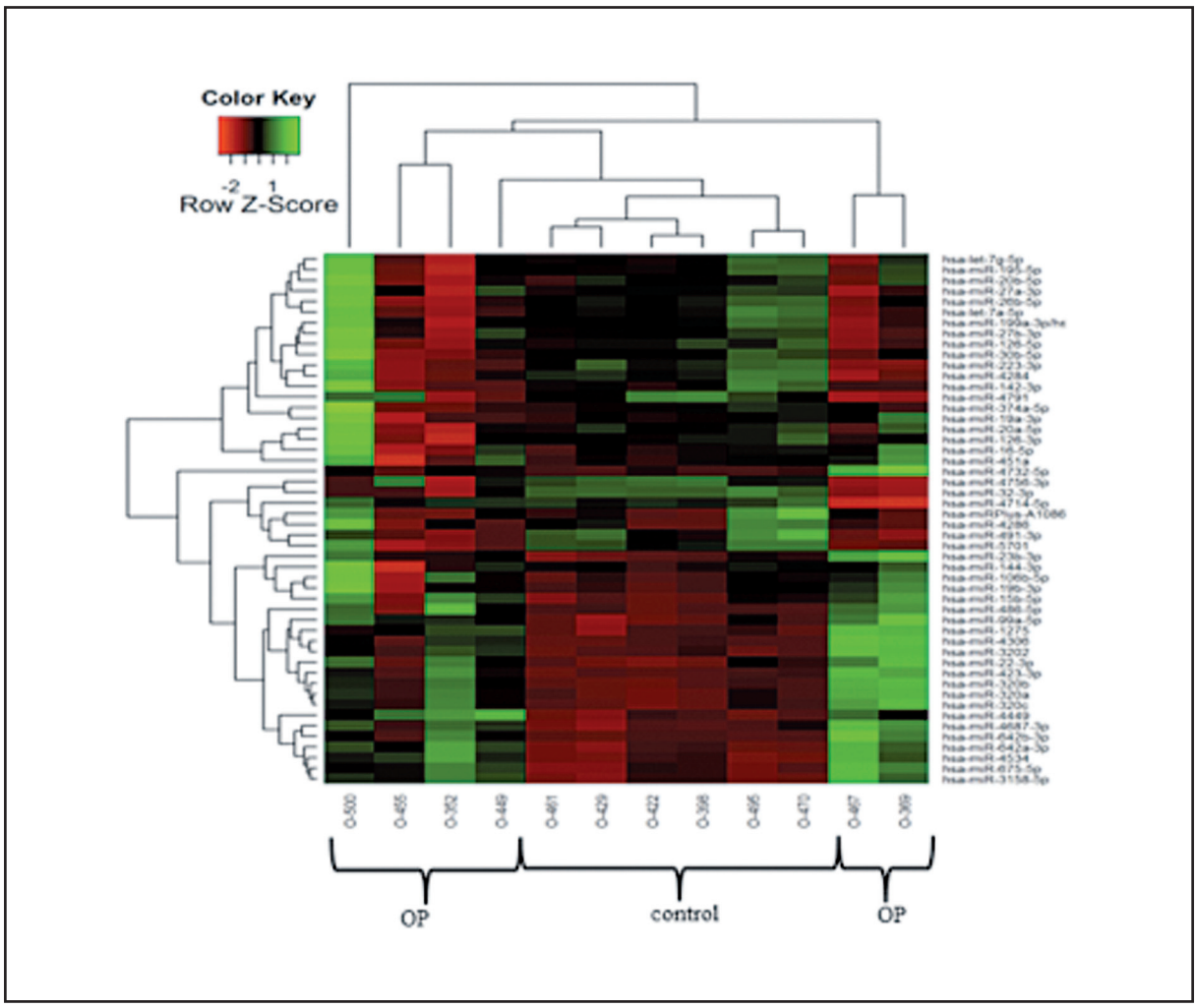

Table 2. MiRNAs significantly altered in samples of osteoporotic bone as microarray results

			Control		OP	
miRNA	**logC**	**P adj val**	**mean**	**SD**	**mean**	**SD**
hsa-let-7a-5p	**-1.819**	**6.99e-03**	**2.94**	**0.78**	**1.12**	**0.64**
hsa-miR-1185-2-3p	1.685	2.37e-02	-1.56	0.15	0.13	1.27
hsa-miR-126-5p	**-1.540**	**2.11e-02**	**2.42**	**0.55**	**0.88**	**0.95**
hsa-miR-1275	2.259	4.90e-03	-1.64	0.41	0.62	1.16
hsa-miR-1307-5p	1.552	4.81e-02	-1.40	0.13	0.15	1.37
hsa-miR-142-3p	-1.850	2.77e-02	2.14	1.22	0.29	0.52
hsa-miR-1470	1.690	5.85e-04	-0.58	0.15	1.10	0.60
hsa-miR-1915-3p	1.583	1.04e-03	-0.82	0.44	0.76	0.44
hsa-miR-204-3p	1.640	9.09e-03	-1.49	0.44	0.15	0.90
hsa-miR-223-3p	-2.777	3.06e-04	2.91	0.67	0.13	0.53
hsa-miR-22-3p	**2.269**	**3.96e-02**	**-1.73**	**0.63**	**0.54**	**1.81**
hsa-miR-25-3p	**1.557**	**1.73e-02**	**-0.90**	**0.24**	**0.65**	**1.06**
hsa-miR-26b-5p	**-2.001**	**6.56e-03**	**3.26**	**0.62**	**1.26**	**0.95**
hsa-miR-30c-1-3p	**2.151**	**3.06e-04**	**-1.65**	**0.38**	**0.51**	**0.57**
hsa-miR-3149	-1.871	2.53e-04	0.59	0.19	-1.29	0.54
hsa-miR-3158-5p	2.513	1.00e-03	-2.00	0.49	0.51	0.91
hsa-miR-3162-3p	1.984	1.64e-03	-1.21	0.22	0.78	0.87
hsa-miR-3178	2.004	7.96e-03	-1.97	0.65	0.04	0.98
hsa-miR-3182	-1.855	3.42e-03	1.73	0.42	-0.12	0.83
hsa-miR-3195	1.795	3.68e-04	-1.50	0.18	0.30	0.57
hsa-miR-3202	2.255	1.42e-02	-1.47	0.24	0.78	1.52
hsa-miR-320a	**1.895**	**2.07e-02**	**-1.39**	**0.33**	**0.51**	**1.35**
hsa-miR-320b	2.085	1.57e-02	-1.61	0.25	0.48	1.42
hsa-miR-320c	1.985	1.98e-02	-1.44	0.34	0.55	1.39
hsa-miR-320d	1.757	2.69e-02	-1.11	0.34	0.65	1.32
hsa-miR-320e	1.687	2.34e-02	-1.21	0.17	0.48	1.26
hsa-miR-32-3p	-2.213	9.00e-04	0.65	0.21	-1.56	0.87
hsa-miR-339-5p	**1.687**	**1.86e-02**	**-1.30**	**0.29**	**0.39**	**1.16**
hsa-miR-3591-5p	-1.511	1.42e-05	0.65	0.11	-0.86	0.20
hsa-miR-3607-3p	-1.853	4.67e-04	0.63	0.31	-1.22	0.55
hsa-miR-3607-5p	-1.508	3.06e-04	1.25	0.33	-0.26	0.30
hsa-miR-3609	-1.542	1.43e-04	1.36	0.34	-0.18	0.19
hsa-miR-361-3p	1.565	2.49e-03	-0.56	0.23	1.00	0.71
hsa-miR-3621	1.806	8.42e-04	-1.96	0.43	-0.16	0.54
hsa-miR-3654	-1.939	2.37e-05	0.39	0.12	-1.55	0.37
hsa-miR-423-3p	**2.081**	**2.90e-02**	**-1.64**	**0.21**	**0.44**	**1.64**

Table 2. MiRNAs significantly altered in samples of osteoporotic bone as microarray results *(cont.)*

			Control		OP	
miRNA	**logC**	**P adj val**	**mean**	**SD**	**mean**	**SD**
hsa-miR-4258	2.034	8.19e-04	-0.56	0.29	1.47	0.74
hsa-miR-4284	-2.957	1.04e-03	2.98	0.81	0.02	0.88
hsa-miR-4306	2.273	2.74e-02	-1.42	0.20	0.85	1.78
hsa-miR-4317	2.177	5.45e-04	-0.81	0.18	1.36	0.78
hsa-miR-4449	2.358	1.32e-03	-2.12	0.33	0.24	0.97
hsa-miR-4455	-1.674	1.51e-03	0.47	0.17	-1.21	0.72
hsa-miR-4458	-1.541	6.59e-04	0.36	0.18	-1.18	0.54
hsa-miR-4463	1.610	4.13e-03	-1.26	0.16	0.35	0.83
hsa-miR-4484	2.002	6.17e-03	-2.00	0.52	0.00	1.01
hsa-miR-4497	1.741	1.57e-02	-1.77	0.48	-0.03	1.08
hsa-miR-4516	1.632	6.14e-03	-0.97	0.36	0.66	0.85
hsa-miR-4532	1.836	1.78e-02	-1.81	0.48	0.03	1.19
hsa-miR-4534	2.278	1.00e-03	-1.80	0.38	0.48	0.86
hsa-miR-4540	-1.547	1.01e-02	0.58	0.14	-0.97	0.97
hsa-miR-4640-3p	1.668	3.06e-04	-1.05	0.21	0.62	0.49
hsa-miR-4687-3p	2.421	7.48e-03	-2.05	0.53	0.38	1.33
hsa-miR-4732-3p	-1.772	5.61e-03	0.36	0.59	-1.41	0.75
hsa-miR-4741	1.666	4.41e-03	-1.44	0.36	0.23	0.79
hsa-miR-4792	1.624	2.11e-02	-1.59	0.31	0.04	1.15
hsa-miR-483-5p	**1.846**	**8.76e-03**	**-1.37**	**0.39**	**0.47**	**1.06**
hsa-miR-491-3p	**-2.889**	**5.68e-03**	**0.79**	**1.43**	**-2.09**	**0.45**
hsa-miR-519e-5p	1.674	6.33e-03	-1.53	0.23	0.14	0.93
hsa-miR-542-5p	2.211	8.30e-04	-1.84	0.42	0.37	0.75
hsa-miR-5681b	-1.513	1.39e-02	0.33	0.54	-1.19	0.82
hsa-miR-5684	-1.710	2.07e-02	0.79	0.18	-0.92	1.24
hsa-miR-5701	-3.127	3.06e-04	0.52	0.76	-2.61	0.63
hsa-miR-574-5p	**-1.552**	**1.31e-04**	**0.52**	**0.16**	**-1.03**	**0.37**
hsa-miR-631	**1.625**	**8.98e-04**	**-1.74**	**0.19**	**-0.11**	**0.61**
hsa-miR-642a-3p	2.530	2.30e-03	-2.13	0.40	0.40	1.15
hsa-miR-642b-3p	2.087	1.67e-02	-1.97	0.15	0.11	1.46
hsa-miR-664b-5p	-1.702	9.09e-03	0.75	0.25	-0.95	1.03
hsa-miR-675-5p	2.323	1.04e-03	-1.85	0.46	0.48	0.84
hsa-miR-711	1.826	2.10e-03	-1.72	0.38	0.10	0.76
hsa-miR-99a-5p	**1.964**	**8.70e-03**	**-1.24**	**0.50**	**0.73**	**1.09**
hsa-miR-99b-5p	**1.706**	**8.81e-03**	**-1.06**	**0.28**	**0.65**	**1.01**

Bold miRNAs that were chosen for validation by qPCR are marked.

Moreover, miR-22-3p is a serum miRNA that was previously associated with osteoporotic fracture[9] and has been implicated in the osteogenic differentiation[18].

Our study results, along with other work in this field, provide an important understanding of bone biology and the involvement of miRNA in the pathology of osteoporosis.

Conclusions

We have identified two miRNAs that are overexpressed in trabecular bone samples from patients with osteoporosis. The expression of both miRNAs is detected in primary osteoblasts, although this overexpression has not been observed in cultures from osteoporotic samples. It is not known whether the alteration of these miRNAs is a cause or effect of the disease and its relationship with osteoporotic fracture. However, these miRNAs could offer promising potential for designing new drugs for osteoporosis.

Funding: This paper was supported by the Thematic Network for Cooperative Research in Aging and Fragility (RETICEF; RD12 / 0043/0022), and support FIS PI13 / 00116 (Carlos III Health Institute, Ministry of Science and Innovation) and FEDER.

Conflict of interests: The authors declare no conflict of interest in relation to this work.

Figure 3. Principal component analysis (PCA) of all samples of the validation phase (OP: n=6, control: n=6). The normalized values (DCQ) were used for analysis. Samples were grouped according to their biological group; however, the O-567 sample appears to be atypical

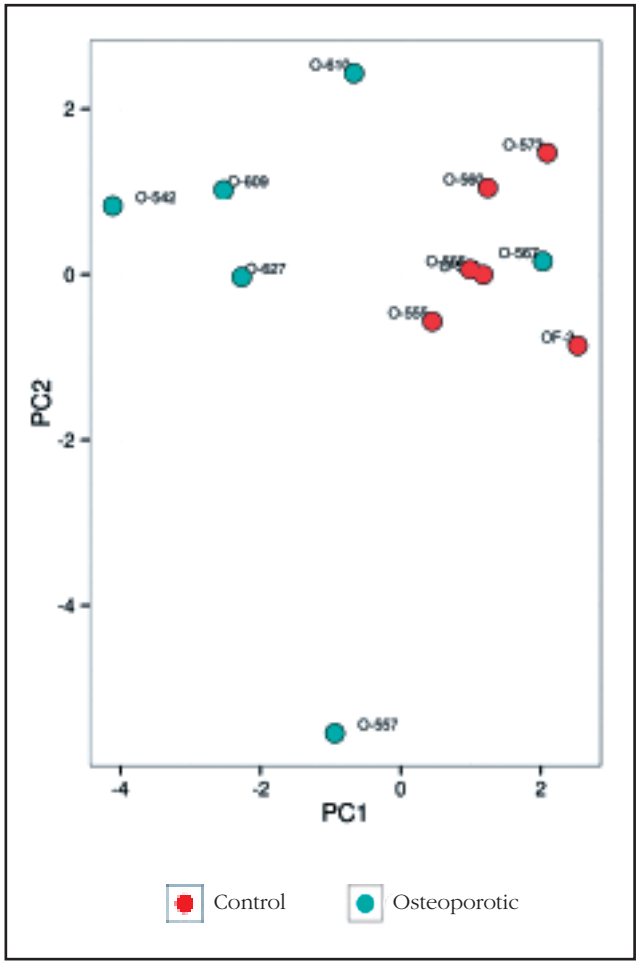

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Table 3. MicroRNAs altered samples with osteoporotic fracture

miRNA	dCq OP	SD OP	dCq control	SD control	2^ddCq	Value p	
						Proof T	Wilcoxon
miR-99a-5p	-1.25	0.53	-0.08	0.21	-2.24	0.01	0.00
miR-339-5p	-1.52	0.23	-1.06	0.22	-1.38	0.01	0.01
miR-320a	2.08	0.98	0.67	0.44	2.65	0.03	0.02
miR-22-3p	1.46	0.37	0.94	0.34	1.44	0.04	0.05

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Medical professionals' perceptions regarding therapeutic adherence in patients with osteoporosis

DOI: http://dx.doi.org/10.4321/S1889-836X2016000100003

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Date of receipt: 08/10/2015
Date of acceptance: 27/11/2015

Summary

Introduction: Adherence to oral treatment of patients with osteoporosis is low, with a high dropout rate in the first year. The most noteworthy result is the lack of therapeutic response.

Objective: To ascertain the perception of physicians working with osteoporotic patients regarding adherence of these patients.

Methods: Cross-sectional study conducted by opinion survey aimed at primary care physicians and specialists involved in osteoporosis treatment. Participants were selected by purposive sampling.

Results: The questionnaire was answered by 235 specialists encompassing rheumatology (54.5%), orthopedics (10.6%) and primary care (18.7%). In 43.8% of participants, more than 25% of patients sometimes forget to take their treatment. According to 34.9%, more than 75% of patients are aware of treatment. Side effects and management complexity are the majority reasons that lead to a change in medication, mean value of 7.94±2.06 6±2.01 points respectively on a 0-10 scale.

Conclusions: Overall, medical specialists attributed low adherence to side effects, polypharmacy and lack of communication between professionals. Dosage and space use of soluble dosage forms may be options to facilitate patient adherence to treatment with oral bisphosphonates. Improved education concerning the importance of the disease or increased patient monitoring could foster adherence.

Key words: *osteoporosis, surveys, bisphosphonates, therapeutic adherence, opinion.*

Introduction

Osteoporosis is a systemic skeletal disease characterized by low bone mass and altered bone microarchitecture causing increased fragility and consequently increased susceptibility to fractures[1]. According to the WHO diagnostic criteria, about 6% of men and 21% of women aged 50-84 years suffer osteoporosis[2]. At a European level, approximately 27.6 million men and women suffered from osteoporosis in 2010, of which 9% were Spanish. Osteoporosis is a major public health problem due to high predisposition to suffer bone fractures[3,4]. Osteoporosis causes more than 8.9 million fractures annually[5], with high healthcare costs[3,6] and a significant decline in the patient's quality of life[2].

The main objective in treating osteoporosis is to prevent fractures, improve patients' quality of life and ease the pain when it occurs. Most of the drugs available today obtain fracture risk reductions of 50-70% for vertebral fractures and 15-25% for the rest of vertebral fractures[2]; provided that the patient takes the medication continuously for the period of time that most baseline studies have shown effectiveness, between 3 and 5 years. Bisphosphonates are the most commonly used alternative therapy in the management of osteoporosis and are considered the first choice in our sector[7,8].

The term adherence encompasses the concepts of compliance and persistence. Compliance involves when and how the prescribed medication is taken, while persistence refers to how long the patient takes it. On the other hand, drug tolerability concerns the patient acceptance of the medication, based mainly on the perception and impact of the drug's unwanted side effects[9].

As osteoporosis is a silent disease, with no symptoms, even in the case of asymptomatic vertebral fractures, patients tend to think that drug treatment is not necessary. On the other hand, the lack of adherence and poor compliance are determined by other factors such as the drugs' side effects, advanced age of patients, polypharmacy or even fatigue patient to take medication on a long-term basis[10].

Adherence to treatment among patients with osteoporosis is low, with a high percentage of dropouts during the first year[11-15]. The most striking result is the lack of therapeutic response and the consequent increase in fracture. So proper adherence to treatment is not only beneficial to patients' health, but also effective in terms of cost-effectiveness[16-20].

This study aims to determine the perception of medical professionals involved in the treatment of osteoporosis concerning patient adherence to treatment in general and in particular regarding bisphosphonates, as well as analyze possible causes and solutions.

Material and methods

This cross-sectional surveyed primary care physicians (PCP) and specialized care professionals who treat patients with osteoporosis. The survey consisted of 13 questions on health professionals' perception regarding adherence of osteoporotic patients (Annex 1), and was completed through a website. Participants were selected through purposeful sampling and invited by the Spanish Society for Bone and Mineral Metabolism Research (SEIOMM) to which they were associated. To estimate the number of specialists participating in the survey, reference was made to a national population of about 20,000 PCP doctors, 5,000 primary care physicians and medical specialists were selected. According to the calculated sample size, made taking the scenario of worst participation ratio to an expected accuracy of 10% and a confidence level of 95% required a minimum of 200 participating physicians.

Statistical analysis was performed using SPSS version 23.0. (SPSS Inc. Chicago, Illinois, USA). The number and percentage of response was used for the description of categorical variables. The mean, standard deviation, median, minimum and maximum are used to describe continuous variables.

Results

The questionnaire was answered by 235 physicians (63.4% male) with a mean age of 48.77±9.13 years. The most represented specialist areas were rheumatology (54.5%), orthopedics (10.6%) and Primary Care (18.7%). Respondents were from 15 different regional communities, with Andalusia (17.4%), Valencia (14.5%), Catalonia (14.5%) and Madrid (11.9%) showing a greater number of participants. 79.6% reported monthly visits to 100 patients for osteoporosis; the rest, 64.3% visited from 25 to 100 and 15.4% less than 25 patients.

Regarding the perception of physicians consulted on patient adherence to oral treatment for osteoporosis, 43.8% said that more than 25% of their patients sometimes forget to take treatment, although 80.4% reported that nearly half of patients do not take the medication at the recommended hours. 34.9% said that more than 75% of patients are conscious about the need for treatment. However, more than half of the patients stop taking it if they experience discomfort, according to 57.5% of the physicians surveyed (Figure 1).

Among the reasons that cause the lack of adherence, 83.0% of respondents felt that the poor coordination between levels of care is an important factor, mainly due to the lack of communication (41.3%), administrative barriers (15.3%), lack of training (14.0%) and applying different protocols (12.3%).

Regarding the causes for a change in treatment, the doctors surveyed reported that the side effects and management complexity are the main reasons, with an average value of 7.94±2.06 and 6±2.01 points respectively (scale of 1: did not motivate changes, 10: motivated major changes) (Figure 2). On the other hand, they indicated that more than half of patients (57%) were usually involved in the choice of treatment.

Figure 1. Attitude of patients regarding oral treatment for osteoporosis

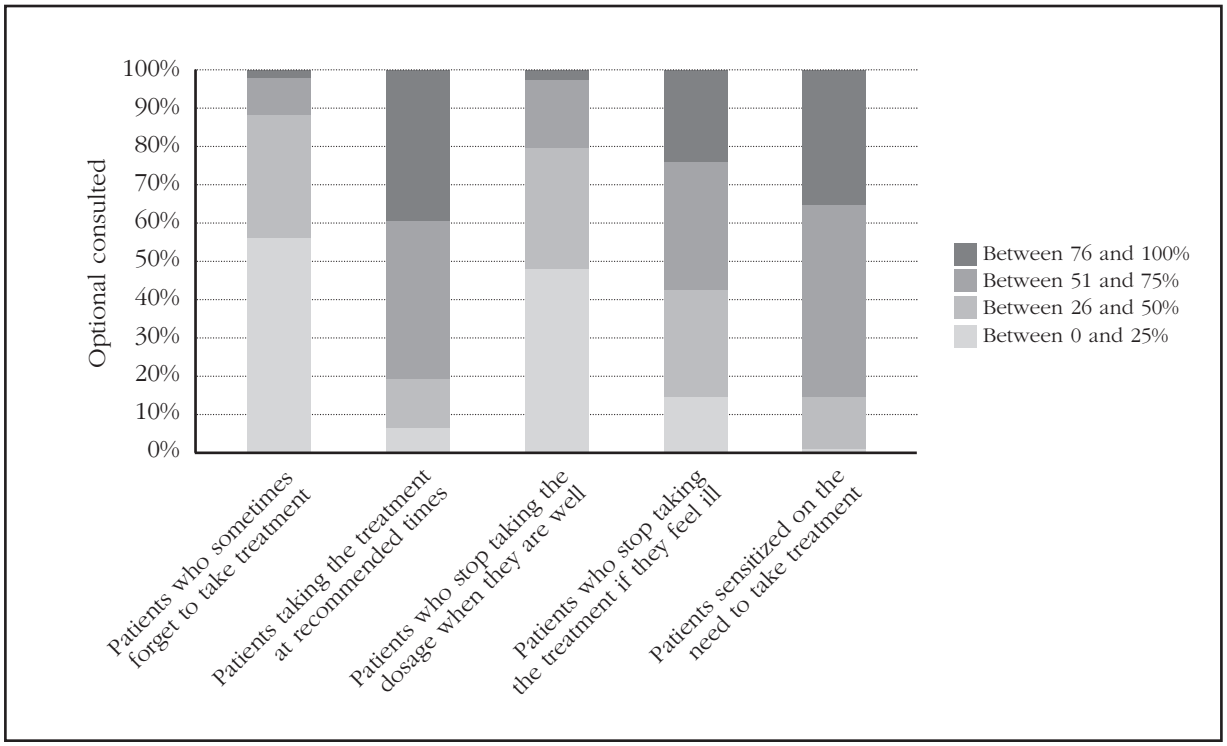
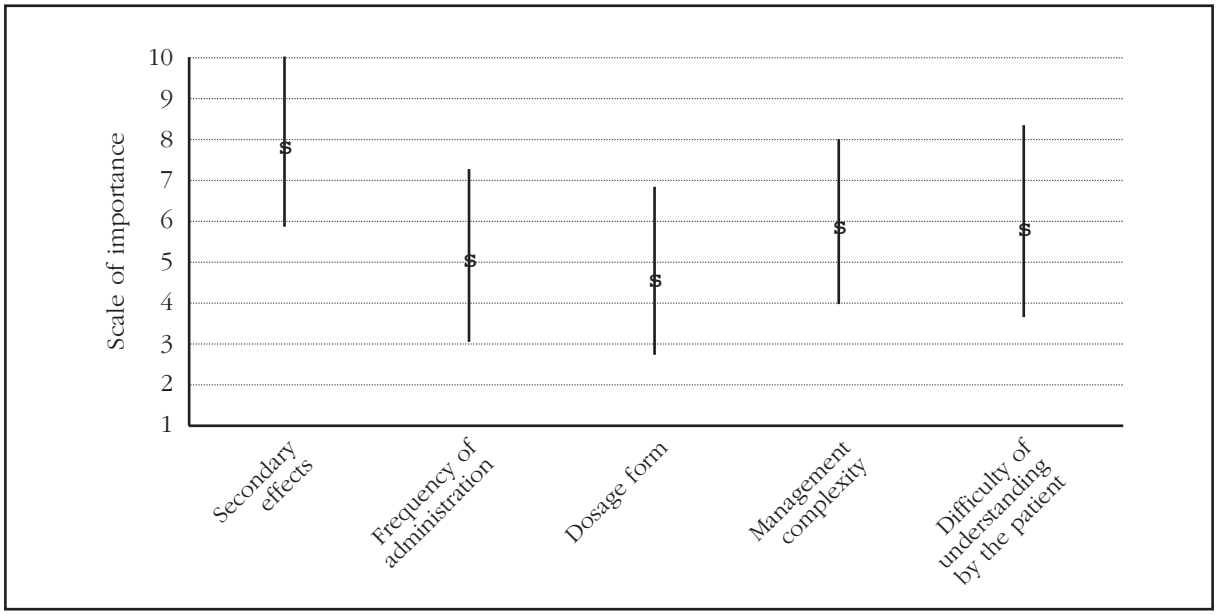


Figure 2. Causes for a change in the oral treatment of osteoporosis

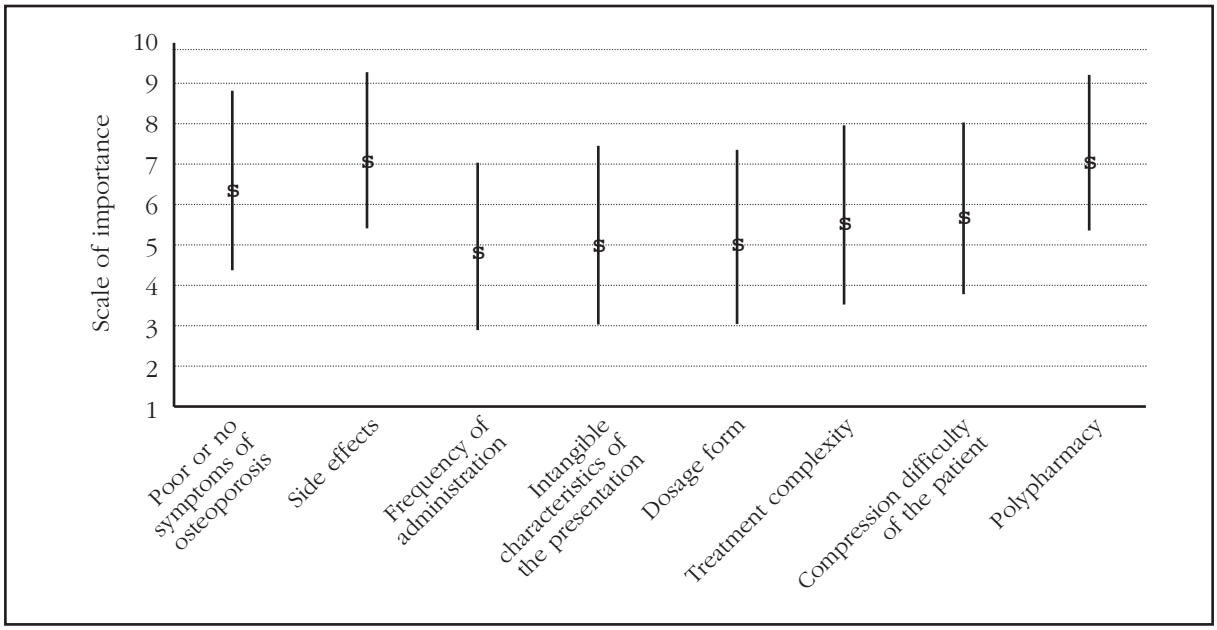


Ascending scale of 1: not motivate changes, 10: motivated major changes.

Regarding the most commonly used methods to assess adherence, 77.9% of respondents reported directly consulting the patient, while 10.2% said that the most common technique is to count the mismatch between the number of containers dispensed or requested by the patient and the amount prescribed. Other methods such as biochemical remodeling markers (4.7%), the Morisky-Green test (3.0%), clinical trial (2.6%) or Haynes-Sackett test (0,9%) were less frequent. Only 0.9% of respondents answered that they did not usually ask about compliance.

Regarding treatment with bisphosphonates, 51-75% of patients are treated and comply with such treatment in 63% and 60.9% of respondents, respectively. Furthermore, among patients who abandon treatment, 40% do so before six months, 29.4% between six and twelve months, and 30.6% after the first year.

Figure 3. Factors causing non-adherence to bisphosphonates



Ascending scale of 1: rare, 10: very common.

According to the physicians surveyed, the main reasons for poor adherence to bisphosphonates are polypharmacy (7.37±1.9 points), side effects (7.34±1.93 points) and the few symptoms of the disease (6.58±2.24 points) (scale of 1: rare, 10: very common) (Figure 3). On the other hand, the restriction of eating and drinking before and after drug intake as instructed is more difficult to follow administration by patients (5.26±2.04 points) (scale of 1: easy to comply, 10: very difficult to enforce) (Figure 4).

As for the impact of various actions to facilitate treatment compliance to bisphosphonates, the most valued (scale of 1: no impact, 10: maximum impact) were: reducing the number of doses (7.57±1.88 points), providing patient with educational material about the disease and its treatment (7.25±1.89 points) and control of adherence in the first few weeks of its inception by nurses (7.12±2.21 points) (Figure 5). Finally, 88.9% of physicians surveyed believed that adherence to oral bisphosphonate treatments would improve greatly or rather a lot if it were administered in a soluble dosage.

Discussion

Regarding monthly care of 100 patients with osteoporosis (79.6% of respondents), and considering that 54.5% of respondents were rheumatology specialists, the results show that, in general, physicians perceive low patient adherence to oral treatment for osteoporosis. The figures concerning compliance and adherence of osteoporotic patients vary among different publications due to the calculation methods used in each. However, all agree that they could be improved[21-23].

The perception of a portion of respondents (43.8%) is that adherence is low, considering that more than 25% of their patients forget to take their medication. These data are consistent with a recent study in primary care centers in the Canary Islands (Spain), where 24.1% of patients with fractures were not taking their prescribed medication[24]. Another retrospective study with similar characteristics performed in Spain showed that 29.5% of patients were not compliant with the proper drug treatments[25].

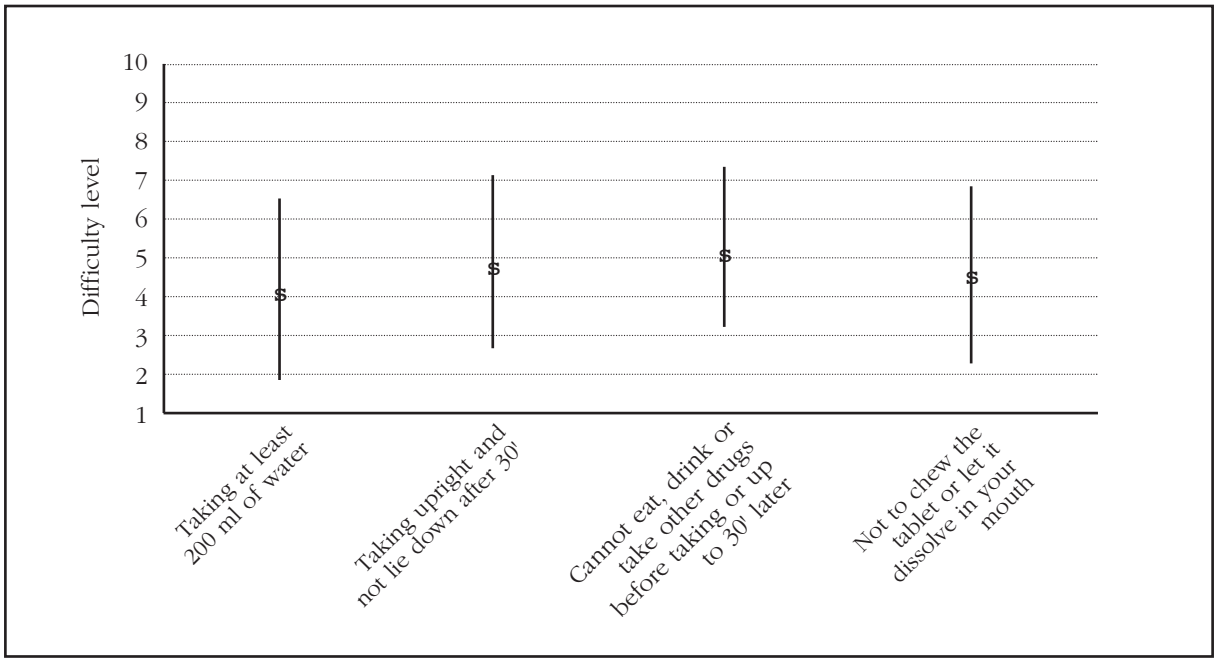
The efficacy of anti-osteoporotic drugs involves prolonged medication, which makes patient neglect of the drug quite common, thus reducing its effectiveness[26]. Clearly, proper adherence is beneficial to patient health[13,16,17,20].

In our study, one of the interesting aspects of the respondents' answers is that among the reasons for lack of adherence, poor coordination between levels of care and lack of communication. There may be communication problems between primary and specialized care, especially at the time of drug prescription, because in many cases the primary care physician is confronted with a medication prescribed by another physician without a specified report. Some studies have already shown that better communication can solve problems better and is a more efficient system[27].

According to respondents, and in line with other publications, other reasons for this lack of patient adherence are the medication's side effects and polypharmacy; which are also perceived as the most common reasons for a change in treatment with bisphosphonates[26,28-32].

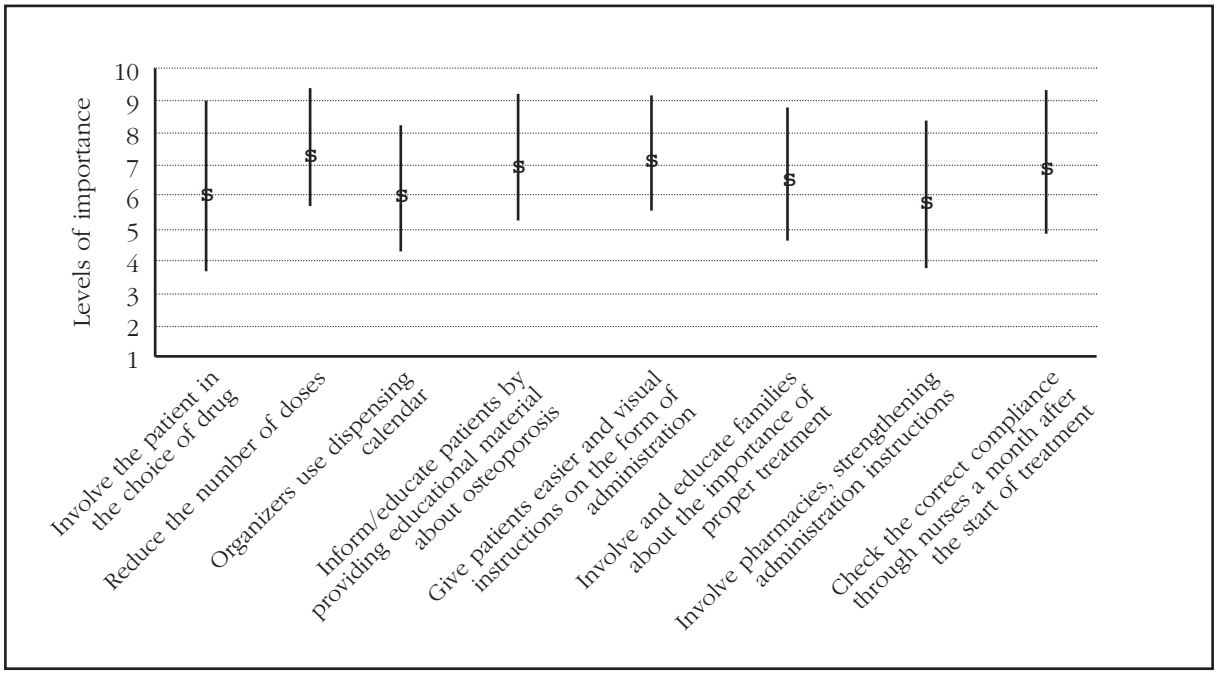
Oral bisphosphonates have become the main drug treatment for osteoporosis[7]. This coincides with the perception of physicians consulted, since, in their view, between 2 or 3 out of 4 patients receiving this treatment present an average level of compliance. However, a high percentage

Figure 4. Assessment of difficulty following instructions for bisphosphonate



Ascending scale of 1: very easy to meet, 10: very difficult to meet.

Figure 5. Actions to improve compliance and correct decision-bisphosphonates



Ascending scale of 1: no impact, 10: maximum impact.

(69.4%) of patients discontinue treatment within the first year, a figure somewhat higher than those reported in other publications[33,34]. In fact, these data reflect the reality of many studies that abandonment of drug treatments and bisphosphonates occurs in 53.9% of cases due to side effects[10].

In this study, polypharmacy and adverse effects seem the main causes of abandonment of oral bisphosphonates. In fact, osteoporosis patients are generally older, and co-morbidity because many of them have received multiple treatments, complicating good compliance and adherence to them. Furthermore, the main adverse effect described with oral bisphosphonates is poor gastrointestinal tolerance, mainly as reflux heartburn or epigastric pain which, as already described in the literature, is one of the main reasons for dropping out.

Assess adherence and treatment compliance require specific tools to ensure methodologic objectivity such as Haynes-Sackett or Morisky-Green tests[9,26]. However, in our study most respondents reported that they preferred direct patient consultation in clinical practice. This reflects the need to improve the query time in both primary care and specialized centers, so that physicians can use more proven methods than simple observation in daily practice.

In line with these study results, the reduced frequency of taking medication, patient education and monitoring of adherence have been proposed among the actions considered that could improve the taking of bisphosphonates[29,30,35-39].

Probably a combination of all these recommendations would be the best strategy to promote compliance and adherence. On the other hand, as most osteoporosis patients are elderly and may have difficulty swallowing, a soluble dosage form would improve the gastric tolerability of bisphosphonates, which would favor patients' treatment compliance, as noted by 88.9% of those physicians surveyed[40].

In conclusion, this survey shows that experts who manage osteoporosis perceived low patient adherence to oral treatment of disease. Poor adherence is mainly embodied by the abandonment of medication during the first year of therapy, and is mainly associated with the side effects, polypharmacy and lack of communication between professionals. Improved comfort by reducing the number of shots and using soluble dosage forms, improved education about the importance of the disease and improved patient follow-up, could foster adherence.

Funding: The study has been funded with a grant from LACER, S.A., who was not involved at any point in the design, analysis, data interpretation, or writing of the final report published manuscript.

Conflict of interest: The authors declare no conflict of interest in this paper.

Annex 1. Additional material: Survey Questionnaire

1. For patients receiving oral treatment for osteoporosis, indicate the percentage:

a) Sometimes forget to take treatments
- 0 - 25%
- 26 - 50%
- 51 - 75%
- 76 - 100%

b) Take the treatments at recommended times
- 0 - 25%
- 26 - 50%
- 51 - 75%
- 76 - 100%

c) Stop taking their treatment doses, when they are well
- 0 - 25%
- 26 - 50%
- 51 - 75%
- 76 - 100%

d) Treatments stop taking them if they are unwell
- 0 - 25%
- 26 - 50%
- 51 - 75%
- 76 - 100%

2. Assess whether the following reasons generate a change in the oral treatment of osteoporosis (rate of 1 to 10, with 10 as motive major changes and a 1 when not cause any changes):

a) Side effects
b) Frequency (daily, weekly, monthly...)
c) The pharmaceutical form (sachets, tablets...)
d) Complexity of administration (fasting, upright position...)
e) Difficulty of understanding by the patient

3. What percentage of your patients that you are aware of the need to take the drugs prescribed think?
- 0 - 25%
- 26 - 50%
- 51 - 75%
- 76 - 100%

4. Do you believe. That one of the causes of poor adherence is the lack of coordination between different levels of care?
- Yes, of administrative impediments
- Yes, by the application of different protocols
- Yes, poor communication
- Yes, lack of training
- No

Annex 1. Additional material: Survey Questionnaire *(cont.)*

5. Are your patients involved in choosing their treatment?

- Yes
- No, because I do not have time
- No, because they have low cultural level
- No, because I leave it to my criteria

From here, we focus on treatment with oral bisphosphonates:

6. What percentage of your patients with osteoporosis are treated with oral bisphosphonates?

- 0 - 25%
- 26 - 50%
- 51 - 75%
- 76 - 100%

7. Of the patients treated with oral bisphosphonates, what percentage comply the treatment?

- 0 - 25%
- 26 - 50%
- 51 - 75%
- 76 - 100%

8. Rate from 1 to 10 the difficulty in compliance for patients with the following instructions for administering oral bisphosphonates (1: very easy to adhere, 10: very difficult to comply):

- It takes at least 200 ml of water
- Take the drug upright and not lie down within 30 minutes of taking it
- Not being able to eat, drink (except for not mineral water) or taking other medication before taking the drug or to at least 30 minutes after
- Not being able to chew the tablet or let it dissolve in the mouth

9. Patients that you control and stop treatment with oral bisphosphonates for osteoporosis ¿how long after having started the treatment do so, on average?

- Before 3 months
- At 3-6 months
- At 6-12 months
- After the first year

10. Assess potential actions that could be taken to improve compliance and correct taking of oral bisphosphonates impact. (1: no impact, 10: maximum impact):

a) Involve the patient in the choice of drug
b) Reduce the number of doses
c) Use dispensing organizers/calendars
d) To inform/educate patients with delivering training material about the disease and the importance of osteoporosis
e) Provide the patient with simple and visual instructions regarding administration
f) Involve and educate families about the importance of correct treatment
g) Involve pharmacies, simplifying the dose instructions
h) Check for correct compliance through nursing staff in the first month of treatment

11. Assess causes for patients with osteoporosis receiving oral bisphosphonates not to abandon treatment. (1: very rare, 10: very common):

a) Poor or no symptoms of osteoporosis
b) Side effects
c) Frequency of administration
d) Presentation organoleptic characteristics (shape, size, hardness, taste, texture...)
e) Pharmaceutical form (envelopes, tablets...)
f) Complexity treatment
g) Difficulty of understanding by the patient
h) Poly-medication (concomitant intake of 6 or more different active ingredients)

12. What method(s) used most frequently to assess adherence to oral therapies for osteoporosis?

- Indirect method of communication self-fulfilling/Haynes-Sackett
- Morisky-Green Test
- Mismatch in the number of packages dispensed/requested by the patient and prescribed
- Direct patient consultation
- Clinical trial
- Biochemical markers of remodeling
- I do not usually ask about treatment compliance

13. Do you think a soluble dosage form will improve adherence to oral bisphosphonate treatments?

- Not at all
- Little
- Quite a lot
- A lot

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Serum dickkopf1 (DKK1), bone metabolism and atherosclerotic disease in patients with type 2 diabetes

DOI: http://dx.doi.org/10.4321/S1889-836X2016000100004

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Date of receipt: 05/10/2015
Date of acceptance: 28/12/2015

Work scholarship from the FEIOMM to attend the 35th Congress of the ASBMR (Baltimore, 2013).

Summary

Background and objectives: Type 2 diabetes (T2DM) is a risk factor for osteoporotic fractures and cardiovascular disease. The aims of our study were to evaluate serum Dickkopf-1(DKK1) levels in a cohort of T2DM patients and to analyze its relationships with bone metabolism and atheroesclerotic disease (AD).

Patients and methods: We studied 126 subjects: T2DM patients (n: 72, mean age 58,2±6 years) and non-diabetic subjects (n: 54, mean age 55,4±7 years). DKK-1 was measured by enzyme-linked immunosorbent assay (ELISA, Biomedica Gruppe). Bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry (DXA). The presence of AD (cerebrovascular disease, peripheral arterial disease, ischemic heart disease) was recorded. Intima-media thickness (IMT) was determined by doppler ultra- sonography and aortic calcification by evaluation of lateral view conventional X-rays.

Results: We did not find significant differences in DKK1 between groups. Serum DKK1 concentrations were significantly higher in females in total sample (24,3±15,2 vs 19,6±10,2 pmol/L, p=0,046) and in T2DM group (27,5±17,2 vs 19,8±8,9 pmol/L, p=0,025). There was a positive correlation between serum DKK1 and LS BMD in total sample (r=0,183, p=0,048). However, we did not find a significant relationship with osteoporosis diagnosis or morphometric vertebral fractures. Serum DKK1 was significantly higher in T2DM patients with AD (26,4±14,5 pmol/L vs 19,1±11,6 pmol/L, p=0,026) and also in patients with abnormal IMT (26,4±15,1 pmol/L vs 19,8±11,3 pmol/L, p=0,038). In the ROC curve analysis to evaluate the usefulness of DKK-1 as a marker for high risk of AD, the area under the curve was 0,667 (95% confidence interval: 0,538-0,795; p=0,016). A concentration of 17,3 pmol/L or higher showed a sensitivity of 71,4% and a specificity of 60% to identify an increased risk of AD.

Conclusions: Circulating DKK1 levels are higher in T2DM with AD and are associated with an abnormal IMT in this cross-sectional study. DKK1 may be involved in vascular disease of T2DM patients.

Key words: *serum Dickkopf1, bone metabolism, atherosclerotic disease, type 2 diabetes mellitus.*

Introduction

Type 2 diabetes mellitus (T2DM) has been linked to an increased risk of fractures at any site despite increased bone mineral density (BMD)[1,2]. Furthermore, atherosclerosis is the major pathogenic mechanism in patients with diabetic macrovascular disease related to a thickening of the arterial wall, the development of the atheromatous plaque and vascular calcification[3]. Atherosclerotic vascular disease is more common in patients with osteoporosis, suggesting several common pathophysiological pathways[4]. In addition, epidemiological data support a relationship between low BMD and the presence of advanced atherosclerotic disease in T2DM[5,6].

Wnt signaling pathways are involved in various physiological processes including cell and tissue differentiation along with morphogenesis of organs[7]. The discovery of the Wnt signaling pathway and its relevance in bone homeostasis has contributed to a better understanding of the cellular and molecular mechanisms of bone biology8. This pathway activation results in expansion of osteo-progenitor cells and a reduction in osteoblast apoptosis, leading to anabolic effects on bones[8]. The canonical Wnt pathway is regulated by multiple families of antagonists, such as Dickkopf-1 (DKK1). DKK1 regulates Wnt signaling by binding to a co-receptor, linked to low density lipoprotein (LRP) 5/6. Furthermore, DKK1 binds to other molecules, such as Kremen transmembrane proteins, to increase its inhibitory activity of the Wnt pathway[9]. The relationship between serum DKK1 and bone mass has been analyzed with contradictory data[10-12].

Given the inverse relationship between bone fragility and atherosclerosis, the role of the Wnt signaling pathway in the process of atherosclerosis is being researched. In preclinical studies, the Wnt signaling pathways are involved in the process of vascular calcification[13], inflammation[14], monocyte adhesion and trans-endothelial migration[15]. There are also recent data that show a relationship between serum levels of DKK1 and atherosclerosis in humans[16,17].

In this context, the objectives of our study were to evaluate serum DKK1 levels in a cohort of patients with T2DM and analyze its relationship with bone metabolism and atherosclerotic disease. In addition, serum DKK1 concentrations in T2DM and non-diabetic subjects were compared.

Patients and methods

Study Population

Our cross-sectional study included 126 subjects: a group of 72 T2DM patients diagnosed with diabetes according to the criteria of the American Diabetes Association (ADA, 2005) and a control group of 54 non-diabetic subjects consecutively recruited from the general population randomly in the same time period.

All study subjects met the following inclusion criteria: Caucasians, outpatient, aged between 35 and 65 and normal blood count, creatinine, liver function, calcium and phosphorus. Exclusion criteria were chronic disease except T2DM situations and treatment with drugs affecting bone metabolism. Those diabetic patients treated with thiazolidinedione were also excluded.

T2DM patients were classified into two groups according to the presence or absence of atherosclerotic disease (AD): AD group and the non- AD group, respectively. Inclusion criteria for patients with atherosclerotic disease were: cerebrovascular disease (ischemic stroke or transient ischemic attack); coronary heart disease (previous myocardial infarction, diagnosis of stable or unstable angina or coronary revascularization) or peripheral arterial disease.

The study was conducted with the approval of the Ethics Committee of the Hospital and adjusted to the relevant guidelines for human research. All patients gave informed consent to be included.

Clinical evaluation

For all patients, height, weight and waist circumference at baseline were measured according to standard procedures. BMI was calculated by dividing weight by the square of height (kg/m^2).

Blood pressure was taken in a standardized way. After 5 minutes of resting blood pressure was measured twice using a standard mercury sphygmomanometer (12 cm long, 35 cm wide). The average of the two values was used for analysis. Hypertension was defined at values ≥140/90 mmHg and/or antihypertensive treatment.

Participants reported their consumption of alcohol, tobacco and level of physical activity in a specific health questionnaire.

Analytical determinations

The plasma glucose level (GBP), glycosylated hemoglobin (HbA1c), calcium, phosphorus and serum creatinine were measured using automated laboratory techniques. High density lipoprotein (HDL), low density lipoproteins (LDL) and triglycerides were measured by standard biochemical methods. Dyslipidemia was defined as the 3rd Report of the Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (ATP-III) or had treatment with statins.

The 25-hydroxyvitamin D (25-OH-D; RIA Kit: DiaSorin, Stillwater, Minnesota, USA); serum parathyroid hormone (Roche Diagnostics SL, Barcelona, Spain immunoassay PTH) were determined.

Markers of bone formation remodeling collected were osteocalcin (OC; RIA Kit, DiaSorin, Stillwater, Minnesota, USA) and bone alkaline phosphatase (BAP; assay enzyme-linked immunosorbent - ELISA-, Tandem-R Ostase TM, Hybritech Europe, Liège, Belgium). Resorption markers included 5β tartrate-resistant acid phosphatase (TRAP5β; colorimetry, Hitachi 704 Boehringer Mannheim GmbH) and carboxy-terminal telopeptide of type 1 collagen (CTX; enzyme immunoassay analyzer CrossLaps Elecsys, Roche Diagnostics SL, Barcelona, Spain).

Serum levels of DKK1 were measured by ELISA (Biomedica Medizinprodukte GmbH and Co. KG, Vienna, Austria) according to manufacturer's instructions. The Biomedical DKK-1 ELISA (BI-20412) detects DKK-1 free. The intra-assay and interassay variability were 7% and 9% respectively. DKK1 measurement is expressed in picomoles per liter (pmol/L).

BMD and vertebral radiographs

BMD of the lumbar spine (CL) L2-L4, femoral neck (CF) and total hip (CT) was determined in all patients by dual X-ray absorptiometry (DXA) using a Hologic QDR 4500 (Whatman, MA; variation coefficient <1%). All measurements were made by the same operator. We use the criteria of the World Health Organization (WHO) for the diagnosis of osteoporosis. plain radiography X-thoracic and lumbar spine for the analysis of vertebral fractures (VF) morphometric rays was also performed, and interpreted according to the algorithm developed by McCloskey et al.[18].

Measurements of intima-media thickness and aortic calcification

The thickness of the carotid intima-media (IMT) was measured by Doppler ultrasound (TOSHIBA Vision 6000) in both carotid about 10 mm proximal to the carotid bifurcation (BIF) with 7.5 MHz probe mode B. The determination He performed by the same observer in all subjects. 10 measurements were performed on each carotid calculating the average for each artery and in turn the average of the two. It is expressed in millimeters, and pathological ≥0,9 mm GIM if defined, and the presence of atherosclerosis if GIM plate ≥1,2 mm or above 50% of adjacent GIM[19].

The presence of aortic calcification was evaluated by radiology simple lateral thoracic and lumbar spine (T4-L5)[20].

Statistical analysis

Statistical analysis of data was carried out using SPSS (version 18.0, Chicago, USA). For continuous variables, it was assessed whether these followed a normal distribution using the Kolmogorov-Smirnov test. Measures of central tendency (mean) and dispersion (standard deviation -SD-, range) were used for continuous variables, and distribution of absolute and relative frequencies for categorical variables. The differences for the variables of interest between comparison groups were performed using Student's t test for independent samples and the test of Mann-Whitney in the case of continuous variables. For categorical variables, the Pearson's chi square and Fisher's exact test were used. The relationship between quantitative variables was analyzed using bivariate correlation test of Pearson or Spearman. The usefulness of serum DKK1 as a marker of high risk for atherosclerotic disease in T2DM was analyzed using a ROC curve (receiver operating characteristic). All statistical tests were double tail. A $p<0.05$ was considered statistically significant.

Results

The clinical characteristics of the study population are summarized in Table 1.

No significant DKK1 differences between the two groups were found: T2DM, 23.35±13.78 pmol/L vs nondiabetic 11.86±20.1 pmol/L, p=0.163. Serum concentrations of DKK1 were significantly higher in women in the total sample (24.3±15.2 vs 19.6±10.2 pmol/L, p=0.046) and in the T2DM group (27.5±17.2 vs 19.8±8.9 pmol/L, p=0.025).

For bone metabolism, there was a positive correlation between DKK1 and lumbar BMD in the total sample (r=0.183; p=0.048). However, no differences depending on the diagnosis of osteoporosis or presence of morphometric vertebral fractures were found. There was also no relation to calciotropic hormones and markers of bone remodeling.

Table 2 shows AD data listed in the groups studied.

DKK1 values were significantly higher in patients with T2DM and AD (26.4±14.5 pmol/L vs 19.1±11.6 pmol/L, p=0.026) and in patients with abnormal GIM (26.4±15.1 pmol/L vs 19.8±11.3 pmol/L, p=0.038).

In the analysis of the ROC curve to evaluate DKK1's utility as a marker of high AD risk, the area under the curve was 0.667 (confidence interval - IC 95%: 0.538 to 0.795; p=0.016). A concentration of 17.3 pmol/L or higher showed a sensitivity of 71.4% and a specificity of 60% for identifying increased AD risk.

Discussion

There are few studies on the relationship between DKK1 and bone metabolism in T2DM. Our results showed higher DKK1 levels in diabetic patients with AD and pathological IMT. These findings suggest that serum DKK1 may be a predictor of the presence of atherosclerotic disease in this population. However, our data showed no differences in DKK1 between diabetic and non-diabetic subjects. For bone metabolism, serum DKK1 significant relationship with bone mineral density was found, while there was none with bone turnover markers, diagnosis of osteoporosis or the presence of morphometric vertebral fractures.

No previous work focused on evaluating the differences in serum DKK1 in accordance with the presence of diabetes. For our part, we found no differences in DKK1 in patients with T2DM and patients without diabetes. These results contrast with our previous data showing high concentrations of sclerostin in this group of diabetic patients[21]. However, the relationship between sclerostin and DKK1 has not been clearly established[22]. Unlike our previous results on sclerostin, women had higher concentrations of DKK1 in both the total sample and in the group of T2DM. The estradiol and progesterone regulate Wnt pathways in endometrial tissue[23] and the brain[24], so the effects induced by sex steroids could explain the gender differences in DKK1.

As for the relationship between bone metabolism and DKK1 we found only a weak correlation with BMD at the lumbar spine in the total sample, and no relationship with markers of bone remodeling, osteoporosis or morphometric vertebral fractures. Furthermore, the lumbar BMD can be affected by aortic calcification. Our findings confirm previous data showing no connection between DKK1 and markers of bone turnover in postmenopausal osteoporosis patients[11] and hemodialysis[12]. The association between DKK1 and BMD is not fully accepted. No relationship was found with BMD in Afro-American diabetic patients[17]. However, an inverse relationship between DKK1 and BMD and higher concentrations of DKK1 in patients with osteoporosis[10] and chronic kidney disease has been reported[22]. Therefore, data on serum DKK1 and bone metabolism are controversial, and prevent drawing clear conclusions.

In our study, the highest levels of DKK1 higher concentrations of DKK1 were related to a diabetic IMT disease were positively related to atherosclerotic disease in patients with type 2 diabetes regardless of the presence of other vascular risk factors. These results are consistent with previous data showing the relationship between vascular disease and DKK1. Patients with cerebrovascular disease have higher serum DKK1 about controls[25] and serum DKK1 correlated with calcification of the coronary arteries and atherosclerotic coronary plaques[16]. Previously, Ueland et al.[26] showed DKK1 gene expression in atherosclerotic carotid plaques and DKK1 is a new mediator of endothelial cell activation mediated by platelets. In contrast to our results, DKK1 concentrations were negatively associated with atherosclerotic plaque in black patients with T2DM[17]. As the authors note, African Americans have a lower prevalence of vascular calcification, and show opposite relationship between arterial calcification and serum concentrations of vitamin D compared to Europeans[27], which could explain the discrepancy of results.

Our study has some limitations as the cross-sectional design does not allow us to establish a cause-effect, and the sample size is relatively small and may affect the finding other interesting results.

In short, DKK1plasma concentrations did not differ according to the presence of diabetes, and we found no relationship with bone turnover markers, diagnosis of osteoporosis or the presence of morphometric vertebral fractures. However, circulating levels of DKK1 are higher in diabetic patients with atherosclerotic disease and related to a pathological IMT. These findings suggest that DKK1 may be involved in the development of atherosclerotic disease in patients with T2DM.

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

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Table 1. Characteristics of the study sample

	Total sample (n=126)	Group T2DM (n=72)	No group T2DM (n=54)	Value p
Age (years)	57±6	58±6	55±7	0.018
Male/female (n)	62/64	39/33	25/29	0.472
Clinic history				
Diabetes duration (years)	-	13.7±7.6	-	
Hypertension (%)	53.2	80.6	46.3	<0.001
Dyslipidemia (%)	65.9	94.4	70.4	<0.001
Tobacco (%)	15.1	16.7	13	0.623
Alcohol (%)	8.7	6.9	11.1	0.104
Sedentary (%)	47.6	55.6	37	0.048
Clinical evaluation				
BMI (kg/m^2)	30.5±5.9	31.4±5.7	29.3±5.9	0.043
Waist (cm)	102.6±12.4	106.4±11.4	97.4±11.9	<0.001
SBP (mm Hg)	130±20	134±97	124±17	0.002
DBP (mm Hg)	80±13	80±12	79±15	0.705
Analytical data				
GBP (mg/dL)	137.2±61.9	173±60.1	89.4±10.4	<0.001
HbA1c (%)	6.7±2.2	8±1.9	4.8±0.4	<0.001
Creatinine (mg/dL)	0.88±0.18	0.89±0.19	0.86±0.16	0.266
Calcium (mg/dL)	9.5±0.5	9.6±0.5	9.3±0.4	0.001
Phosphorus (mg/dL)	3.6±0.5	3.7±0.5	3.5±0.5	0.01
PTH (pg/mL)	43.6±19.5	38.5±18.4	50.4±19.1	<0.001
25(OH)D (ng/mL)	19.5±11.3	17.8±11.5	21.6±10.9	0.06
OC (ng/mL)	1.5±1.3	1.5±1.3	1.5±1.2	0.939
BOP (µg/L)	14±6.5	14.7±6.2	13±6.8	0.162
CTX (ng/mL)	0.266±0.155	0.209±0.132	0.338±0.153	<0.001
TRAP5b (UI/L)	1.6±0.9	1.4±1	1.8±0.8	0.02
Triglycerides (mg/dl)	142±121	169.9±149.8	104.9±47.7	<0.001
HDL (mg/dl)	53.5±15.5	49±16	59.5±12.5	<0.001
LDL (mg/dl)	111.7±35.5	96.9±34.1	130.8±27.4	<0.001
DKK1 (pg/ml)	**21.95±13.1**	**23.35±13.78**	**20.1±11.86**	0.163
DXA parameters and fractures				
BMD LS (g/cm^2)	0.977±0.148	0.954±0.146	1±0.148	0.068
BMD FN (g/cm^2)	0.820±0.124	0.817±0.132	0.823±0.117	0.792
BMD TH (g/cm^2)	0.906±0.135	0.903±0.145	0.911±0.125	0.772
T-score LS	-1.08±1.36	-1.3±1.3	0.82±1.3	0.058
T-score FN	-0.55±1.01	-0.6±1.04	-0.49±0.99	0.565
T-score TH	-0.55±0.98	-0.62±1	-0.51±0.92	0.557
Osteoporosis (%)	15.9	24.6	9.4	0.047
Fractures (%)	23	30.3	20	0.274

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; GBP: basal plasma glucose; HbA1c: glycated hemoglobin; PTH: parathyroid hormone; 25 (OH) D: 25-hydroxyvitamin D; OC: osteocalcin; BOP: bone alkaline phosphatase; CTX: carboxy-terminal telopeptide of type 1 collagen; TRAP5b: 5β tartrate resistant acid phosphatase; HDL: high density lipoprotein; LDL: low density lipoprotein: BMD: bone mineral density; LS: lumbar spine; FN: femoral neck; TH: total hip; IMT: intima-media thickness.

Table 2. Atherosclerotic disease according to study groups

	Total sample (n=126)	Group T2DM (n=72)	No group T2DM (n=54)	Value p
Atherosclerotic disease	35.7	58.3	5.6	<0.001
Cerebrovascular disease (%)	11.9	19.4	1.9	0.002
Cardiopaty (%)	23.8	38.9	3.7	<0.001
Peripheral arterial disease (%)	7.9	13.9	0	0.005
Pathological IMT(%)	35.7	54.2	11.1	<0.001
Carotid plaque (%)	15.9	29.4	0	<0.001
Aortic calcification (%)	19	34.8	2.2	<0.001

IMT: intima-media thickness.

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Changes induced by DKK1 in rheumatoid arthritis patients who commence biologic therapy treatment

DOI: http://dx.doi.org/10.4321/S1889-836X2016000100005

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Date of receipt: 08/10/2015
Date of acceptance: 06/02/2016

Work rewarded with the scholarship of Clinical Investigation FEIOMM 2013.

Summary

Introduction: The aim of this study is to assess the relationship among inflammatory charge, cardiovascular risk and bone metabolism in patients with rheumatoid arthritis initiating biological therapy treatment.

Patients and methods: This is a prospective cohort study conducted in patients diagnosed with active rheumatoid arthritis (RA) assessed in the Rheumatology Unit and initiating biological therapy.
Patients will be selected consecutively, with preliminary data on 14 patients. We present preliminary data from 14 patients.

Results: Reduced Dickkopf-1 (DKK1) concentrations after commencing biological therapy were detected (baseline: 53.12±60.43 pg/ml vs 6 months 13.5±23.2 pg/ml, p=0.307) but without statistical significance. Changes were found in markers for bone remodeling with increased osteocalcin levels and CTX which were not statistically significant either.

Conclusions: We observed a nonsignificant decrease in DKK1 serum in patients with active RA treated with biologic therapy. Expanding the scope of study subjects and pending biochemical determinations will allow us, in the near future, to establish more precisely this link and the relationship of DKK1, bone remodeling, biological therapy and cardiovascular disease in RA patients.

Key words: *rheumatoid arthritis, DKK1, biological therapy.*

Introduction

Rheumatoid arthritis (RA) and other inflammatory rheumatic diseases such as ankylosing spondylitis and psoriatic arthritis have an increased cardiovascular mortality due to accelerated development of atherosclerosis[1]. Persistent chronic inflammation and genetic factors have been associated with the development of accelerated atherosclerosis and consequently cardiovascular events[2].

The Wnt pathway has been involved not only in altering the bone metabolism[3] but also in cardiovascular disorders[4,5], which may be the common link between these diseases. The implication of rheumatoid arthritis in this way explained by pro-inflammatory cytokines involved in its pathogenesis, such as tumor necrosis factor alpha (TNF-α), which plays an important role in the process of osteoclast differentiation by increasing the ligand receptor activator of nuclear factor kB ligand (RANKL) and Dickkopf-1 (DKK1) and sclerostin, both Wnt pathway inhibitors[6]. Thus, control of activity in patients with RA should entail not only an increase in BMD but also reduced cardiovascular risk.

The aim of the study is to assess the relationship of the inflammatory burden, cardiovascular risk and bone metabolism in patients with rheumatoid arthritis who start treatment with biological therapy. To do this, we have analyzed the relationship between inflammatory activity, serum concentrations of antagonists of the Wnt pathway (DKK1), the specific cardiovascular disease by the modified SCORE method for RA, the intima-media carotid and bone disease in patients with RA, at the start of treatment with biological therapy and at 6 and 12 months of treatment. In this paper, preliminary data from the study are presented.

Patients and methods

This is a prospective cohort study of patients diagnosed with active RA evaluated at the Rheumatology Unit, and commence biological therapy. For the diagnosis of RA 1987 ACR criteria were used. Inclusion criteria were the following RA diagnosis, over 18 years of age, presence of disease activity (DAS> 2.4) despite treatment with synthetic disease-modifying drugs and signed informed consent. We excluded patients with previous cardiovascular events, previous osteoporotic fractures, osteoporosis different metabolic bone disease, chronic renal disease, chronic liver disease, type 1 and 2 diabetes mellitus, neoplastic disease, pregnancy and lactation.

The study was approved by the Ethics Committee for Clinical Research of the University Hospital Rafael Mendez. All participants were informed of the type of study and its procedures, and provided informed consent before any study procedure. The study was designed and conducted in accordance with the ethical standards of the Helsinki Declaration.

The following variables were collected: DKK-1 serum levels, sociodemographic characteristics, blood pressure (BP), DAS-28 VSG, visual analogue scale (VAS) of the patient's disease on a 0 to 10 scale, duration of the disease determined in years, response to treatment assessed by EULAR, values for rheumatoid factor and anti-citrullinated peptide antibodies, blood count, general biochemistry with hepatorenal function, lipid profile (total cholesterol, HDL, LDL, triglycerides), C-reactive protein (CRP), and serum calcium, phosphorus parathyroid hormone (PTH), 25-hydroxyvitamin D3 (25-OH vitamin D3), bone turnover markers (bone alkaline phosphatase, osteocalcin, C-terminal telopeptide of type I collagen -u-CTX), thickness intima-media carotid (c-IMT), model SCORE (systematic coronary risk evaluation, systematic coronary risk Assessment) model modified for AR SCORE, and bone mineral density in the lumbar spine and hip measured by dual X-ray absorptiometry (DXA).

Biochemical determinations

The analysis of biochemical parameters was carried out by standard techniques.

Calciotropic hormone concentrations were determined by HPLC for 25-OH vitamin D3 and Elecsys systems for intact PTH. Remodeling biochemical markers were determined in an automated program (Roche Elecsys 2010).

DKK1 concentrations were evaluated by ELISA (Biomedica Medizinprodukte GmbH and Co. KG, Vienna, Austria) following manufacturer's instructions. For all other pending biochemical determinations, frozen samples at -80 ° C were used.

BMD Evaluation

Bone mineral density at the lumbar spine and femoral neck was assessed by dual X-ray densitometry (DXA) (Norland XR-800).

For postmenopausal women and men >50 years, the T-score was used to classify the central DXA in normal, osteopenia and osteoporosis. Z-score was used in the other cases; a Z-score <-2 was considered low bone mass.

Evaluation of c-IMT

The ultrasound evaluation of the carotid arteries was performed using Doppler ultrasound (Philips iU22) with a 9-3 MHz linear probe. The c-IMT was assessed and also the existence of plates. The c-IMT was measured in the distal third of both carotid arteries 1 cm above the bulb. The plate was defined as a greater focal thickening of 0.5mm within the arterial lumen or thickening >50% of the thickness of the adjacent intima or intimal thickness >1.5 mm.

Cardiovascular risk assessment

The patients' cardiovascular risk was determined by the SCORE model and modified to the AR SCORE. Those patients who had carotid ultrasound plates and/or c-IMT >0.9 were classified as patients at very high cardiovascular risk regardless of the SCORE obtained.

Table 1: Sociodemographic and clinical characteristics

N	14
Age, mean ± SD	47.14±14.06
Woman, n (%)	9 (64.3)
BMI, mean ± DE	29.89±7.41
HBP, n (%)	3 (21.4)
DLP, n (%)	3 (21.4)
Alcohol intake, n (%)	2 (14.3)
Smoking, n (%)	9 (64.3)

SD: standard deviation; BMI: body mass index; HBP: high blood pressure; DLP: dyslipidemia.

Statistical analysis
Data for continuous variables are expressed as mean ± standard deviation. The data for categorical variables are presented as percentages. Changes in quantitative variables before and after treatment were compared with Student's t test for paired samples. Categorical variables were compared by chi-square test.

Correlation analyzes were performed using Pearson's correlation (normal distribution) or Spearman (non-normal distribution). P values <0.05 were considered significant. For statistical analysis, the SPSS version 18.0 software (SPSS, Chicago, IL) was used.

Results

As of September 2015 14 naïve patients have been included for biological therapy. In this paper, we present the results at 6 months.

Demographic-clinical variables
The average age of the 14 patients was 47±14 years. 64.3% were women. 21.4% of the patients were hypertensive and 64.3% were smokers. The values of other variables are shown in Table 1.

Variables related disease
The average disease duration was 68±71 months (CI 10-240). The DAS 28-ESR mean baseline was 4.41±1, the average number of swollen joints 3±2, the average number of painful joints 4±3 and visual analogue scale (VAS) of the specific disease by 7±2 patient.

71.5% had positive rheumatoid factor and anti-citrullinated peptide antibodies. 64.3% of patients included in the study were taking disease modifying drugs (DMARDs) associated with biological therapy. The average prednisone dose was 3.7±2.5 mg. Only 44.4% of the patients had EULAR response to treatment at 6 months.

Analytical and variables related to bone metabolism
The values of these variables are represented in Table 2.

Correlation between bone remodeling, DKK1, disease activity and c-IMT
No significant correlation was found between disease activity measurement by DAS 28-ESR and levels of alkaline phosphatase, osteocalcin, CTX or DKK1. Nor did we observe any relationship between markers of remodeling or concentrations of DKK1 and intima-media thickness.

We found no association between DAS28-ESR-measured disease activity and cardiovascular risk assessed by SCORE and modified SCORE.

DKK1 changes after treatment and relationship with disease parameters
We found decreased levels of DKK1 after commencing biological therapy (baseline: 53.12±60.43 pg/ml vs 6 months 23.2±13.5 pg/ml, p=0.307) which was not statistically significant (Figure 1). No statistically significant association between decreased levels of DKK-1 and EULAR response to treatment was detected. As for bone remodeling markers, insignificant increased osteocalcin levels and CTX were detected.

Discussion

Epidemiological studies have shown an association between BMD loss and cardiovascular calcification, morbidity and mortality[7-9]. The Wnt pathway is involved in regulating vascular calcification and differentiation of smooth muscle cells to osteoblasts10. It has been shown to increase the DKK1 expression[11,12] in carotid atherosclerotic plaques and increased serum concentrations of sclerostin in patients with atherosclerotic disease and type 2 diabetes[13].

Furthermore, elevated levels of circulating DKK1 in patients with RA have been shown, linked to radiological damage[14-18], and sclerostin expression seems to correlate positively with levels of DKK1[19].

Our study showed a decrease in the levels of DKK-1 at 6 months of treatment, which is consistent with recently published by Briot et al.[20]. In this article, patients with active RA treated with tocilizumab experienced a decrease in DKK1 concentrations and a decrease in formation markers. However, in our study we found an increase in markers of formation and post-treatment resorption, but this did not reach statistical significance. Our limited sample size has certainly influenced our results.

In conclusion, we can say that in patients with active RA treated with biological therapy we have observed a nonsignificant decrease in serum concentrations of DKK1 and a significant increase in bone resorption. Expanding both the number of study subjects as well as more pending biochemical determinations would allow us in the near future to more precisely establish this association, and also the relationship between DKK1, bone remodeling, biological therapy and cardiovascular disease in patients with RA.

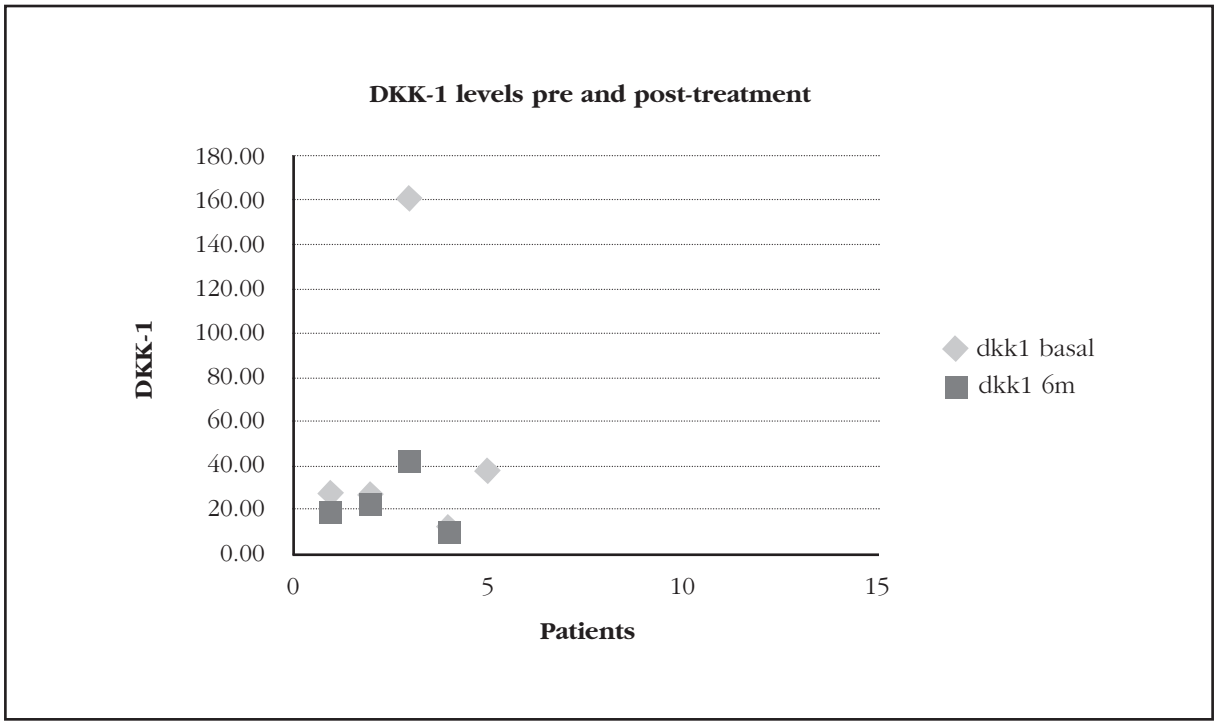
Competing interests: The authors declare no conflicts of interest regarding this article.

Table 2: Biochemical and related bone metabolism, disease activity and baseline cardiovascular risk and at 6 months

	Basal (N=14)	6 months (N= 9)
Total cholesterol (mg/dl), mean ± SD	198.85±37.09	195±55.91
HDL-cholesterol (mg/dl), mean ± SD	62.71±24.32	52±24.06
LDL-cholesterol (mg/dl), mean ± SD	129.57±34.24	138±59.07
Triglycerides (mg/dl), mean ± SD	117.85±43.95	154±76.80
CRP (mg/L), mean ± SD	8.62±10.50	6,66±9.34
IPTH (pg/ml), mean ± SD	37.85±12.76	52.01±14.88
25-OH vitamin D (ng/dl), mean ± SD	18.70±6.89	28.40±19.51
FAO (µg/dl), mean ± SD	11.82±4.20	11.50±4.13
Osteocalcin (ng/ml), mean ± SD	13.73±6.75	19.27± 11.54
CTX (ng/ml), mean ± SD	0.26±0.11	0.37±0.21
DXA central		
- Normal, n (%)	11 (78,6)	
- Osteopenia, n (%)	0 (0)	
- Osteoporosis, n (%)	1 (7,1)	
DAS 28-VSG, mean ± SD	4.41±0.99	3.38±1.51
EVA-disease, mean ± SD	7±2	3.66±2.82
SCORE		
- Low, n (%)	6 (42.9)	5 (55.6)
- Moderate, n (%)	8 (57.1)	3 (33.3)
- High, n (%)	0 (0)	1 (11.1)
- Very high, n (%)	0 (0)	0 (0)
SCORE modified		
- Low, n (%)	6 (42.9)	5 (55.6)
- Moderate, n (%)	6 (42.9)	2 (22.2)
- High, n (%)	2 (14.3)	1 (11.1)
- Very high, n (%)	0 (0)	1 (11.1)
SCORE-ultrasound		
- Low, n (%)	5 (35.7)	5 (55.6)
- Moderate, n (%)	1 (7.1)	2 (22.2)
- High, n (%)	1 (7.1)	1 (11.1)
- Very high, n (%)	7 (50)	1 (11.1)
c-IMT right (mm), mean ± SD	0.54±0.18	0.51±0.10
c-IMT left (mm), mean ± SD	0.64±0.20	0.49±0.08
Carotid plaques, n (%)	7 (50)	2 (25)

SD: standard deviation; CRP: C-reactive protein; FAO: bone alkaline phosphatase; CTX: C-terminal telopeptide of type I collagen; c-IMT: carotid intima-media thickness.

Figure 1. Changes in concentrations of DKK1 after biological treatment



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Classic non-deforming osteogenesis imperfecta. Report of a new mutation in the COL1A1 gene in two cases in the same family

DOI: http://dx.doi.org/10.4321/S1889-836X2016000100006

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Date of receipt: 26/01/2016
Date of acceptance: 16/03/2016

Summary
Osteogenesis imperfecta (OI), is a rare condition which is heterogeneous in clinical and genetic terms. Several types have been described and its main feature is bone fragility. It is generally caused by gene mutations in those genes which codify for the α1 and α2 of the type 1 collagen (COL1A1 and COL1A2) with dominant autosomal heredity.
We report the case of two relatives (father and daughter) with OI whose genetic study shows a mutation in COL1A1 previously undetected: the deletion of a Guanine, G(c.3524delG). Clinical aspects, heredity and reproductive options of the patients affected are considered.

Key words: *osteogenesis imperfecta, genetic research, COL1A1 gene.*

Introduction

Osteogenesis imperfecta (OI), also known as "brittle bone disease", is a rare and very heterogeneous disease from the clinical and genetic point of view. It is due to mutation of genes involved in the formation of type 1 collagen, affects one in every 15,000-20,000 live births. Although its main, common characteristic is bone fragility, several types have been described depending on genetic radiological and clinical features[1-3].

Generally, OI is caused by heterozygous mutations in the genes encoding the α2 and α1 chain of type 1 procollagen (COL1A1 and COL1A2 genes) but other genes have been identified. Mutations in the COL1A1 and COL1A2 genes are inherited in an autosomal dominant pattern[1-4].

We report two cases of two separate patients (father and daughter) affected by a non-deforming type of OI (probably Sillence type 1) in which the genetic study shows a mutation in the COL1A1 gene not previously known. This heterozygous deletion of a guanine, G (c.3524delG), is not described in the literature or in the databases.

Case 1

A 65-year-old male patient diagnosed with OI following a study that showed the daughter was also affected. He showed no previous family history of the disease (Figure 1).

He reported a history of multiple fractures (approximately 6-7) during childhood and adolescence after minor trauma, the first of the humerus at 2 years old. In adulthood he had two new fractures in elbow and shoulder. Both had been treated conservatively. He was treated with calcium during childhood. He had also presented throughout his life several multiple sprains and muscle tears. Diagnosed with otosclerosis, he had undergone stapedectomy surgery of both ears.

He was referred to our clinic for osteoporosis detected in bone densitometry (DXA), which showed a T-scores of -3.4 at the lumbar spine (L1-L4), femoral neck -3 and -2.8 in total femur. It was asymptomatic.

On physical examination, the size of 162 cm was noteworthy as was blue sclera and the presence of dentinogenesis imperfecta. He did not present thoracic, lumbar vertebral column or member deformities, except in the right elbow (post-fracture). No hypermobility was detected.

The study of calcium-phosphorus metabolism showed normal levels of calcium, phosphorus, urinary calcium, and parathyroid hormone (iPTH). Levels of 25-hydroxyvitamin D (25OHCC) were insufficient: 22 ng/ml (desirable values >30 ng/ml). The bone turnover markers were in normal range. other endocrine causes of osteoporosis were excluded.

As part of our study protocol of patients suffering from OI, an X-ray of cervical spine basilar impression was taken and a chest image in which degenerative changes were evident in the column. ECG showed minimal dilatation of the ascending aorta; respiratory function tests were normal, and abdominal ultrasound ruled out nephrolithiasis.

Genetic study was carried out using massive sequencing by NGS (Next-Generation Sequencing) of the COL1A1, COL1A2, and LEPRE1 CRTAP genes, detected in the COL1A1 gene deletion of a guanine heterozygosity (c.3524delG).

This mutation results in a change in the reading frame, which, at the level of collagen protein, results in a premature stop codon (p.Gly1175Valfs*64) so it is likely to be a pathogenic change. Other detected changes were considered polymorphisms.

Weekly treatment was recommended with alendronate along with daily supplements of calcium and vitamin D, showing a slight densitometric improvement after 1 year of treatment (T-scores of -3.2 at the lumbar spine, femoral neck -2.9 and -2, 4 total femur).

Case 2

A 30-year-old patient, daughter of former patient (Figure 1), diagnosed in childhood with OI following a displaced fracture of tibia followed by broken collarbone after minor trauma. She then presented three new fractures, the most recent at age 12, which required surgical treatment (olecranon). Among other relevant history, multiple ankle sprains and right ear stapedectomy were highlighted. She had never been treated for this disease and presented asymptomatic.

The patient requested reproductive data regarding her chances of having a child free of disease.

Physical examination revealed a size of 153 cm, blue sclera and normal teeth. No deformities were observed at any level.

Laboratory tests were normal, except 25OHCC levels: 23 ng / ml, and the rest of the study (cervical spine radiography, echocardiography, spirometry, abdominal ultrasound). The results of densitometry showed normal BMD: T-scores of -0.9 at the lumbar spine (L1-L4), femoral neck and 0.0 - 0.1 total femur. Vitamin D supplements were recommended.

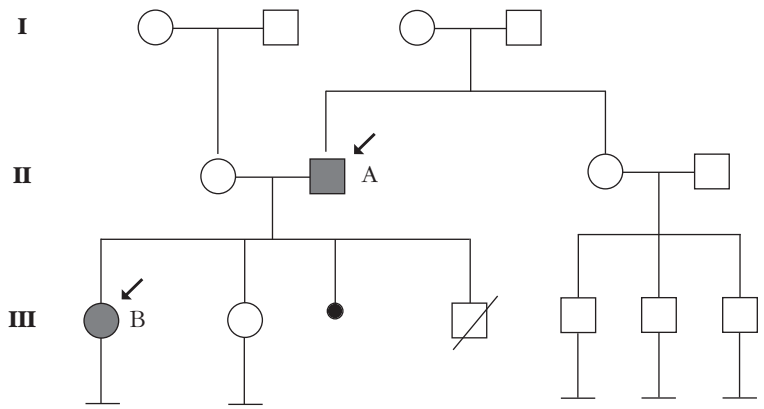
Directed genetic study was performed to search for the identified mutation in her father, confirming that the child carries the same deletion in the COL1A1 gene heterozygosity as her father. Genetic counseling was conducted to report on the possible consequences for affection to offspring, the results of a genetic study and their advantages and risks, and inform them of the possible alternatives derived from the analysis.

Discussion

Type 1 collagen is a structural protein that forms part of the bone, skin, teeth, tendons, ligaments and sclera. In general, OI is caused by heterozygous mutations in the genes encoding the α1 chains and α2 type 1 (COL1A1 and COL1A2 genes) procollagen but also have identified other

Figure 1

Family pedigree with previously undescribed mutation imperfeta causing osteogenesis (OI) in 2 cases



A: Case 1. OI male affection. Heterozygote deletion in COL1A1 gene c.3524delG

B: Case 2. OI female affection. Heterozygous deletion in COL1A1 gene c.3524delG

genes involved in the processing of type 1 collagen, such as CRTAP and LEPRE. The COL1A1 gene is located on chromosome 17 q21 region in-q22, COL1A2 gene and chromosome 7 in the region q22. Mutations in the COL1A1 and COL1A2 genes are inherited in a dominant autosomal pattern[1-4]. That is, every time a parental affection conceives a child, there is a 50% chance in every pregnancy of passing on the disease, regardless of sex.

Clinical abnormalities of OI related to COL1A1 and COL1A2 genes are primarily fractures without trauma or after minimal trauma, variable dentinogenesis imperfecta and hearing loss in adulthood. The severity of the clinical presentation depends on the effect of the mutation. Mutations that bring about a reduction in the amount of synthesized collagen forms are milder than those affecting the proteic structure[5].

There is a continuum from the most severe form, the perinatal-lethal (type 2 Sillence classification), individuals with severe deformities, short stature and impaired mobility (types III and IV Sillence) to patients virtually asymptomatic of dentinogenesis imperfecta normal height and predisposition to fractures, but with normal life expectancy (type I Sillence)[5].

The diagnosis of OI should be based on family history, history of fractures, usually spontaneous or with minimal trauma, short stature, sometimes associated with more or less severe deformities, and the presence of other clinical data such as blue or gray sclera, dentinogenesis imperfecta, ligamentous laxity and progressive hearing loss after puberty. Radiographic findings include osteopenia or osteoporosis, wormian presence of bones, skeletal deformities and fractures or its aftermath. A molecular genetic study is recommended to confirm the diagnosis[6-7].

The two cases described here show a mild form, the most frequent, with common characteristics such as predisposition to fractures with minimal trauma, short stature, blue sclera, ligamentous laxity prone to sprains and dislocations and hearing loss transmission mild form of early OI, but do not share other clinical data such as dentinogenesis imperfecta. In mild forms, it is possible to find a bone mineral density within normal limits because the DXA measures bone mineral and not collagen content[9]. It has been reported that there is no clear genotype-phenotype correlation even within the same family[6].

Genetic studies of genes COL1A1 and COL1A2 detect abnormalities in 90% of individuals with OI types I, II, III and IV of Sillence. Its sensitivity is similar to the quantitative and structural analysis of type 1 collagen in cultured fibroblasts obtained from a skin biopsy[8].

Mutations were found more often in the COL1A1 gene (up to 70% of cases) the COL1A2 gene in both cases and inherited autosomal dominant or behave as de novo mutations. In all, more than 1,500 different mutations have been described. In our patients, the study found a heterozygous deletion of a guanine (c.3524delG) in the COL1A1 gene. This mutation, not previously described in the literature, gives rise to a change in the interpretive framework, which level collagen protein, results in a premature stop

codon (p.Gly1175Valfs*64), so it is very likely a pathogenic change. Other detected changes were considered polymorphisms without clinical association.

De novo mutations constitute 100% of cases with lethal perinatal-(type II Sillence), almost 100% in progressively deforming shapes and about 60% of non-classical deformities[6]. For the family history of our patients, the father suffered a de novo mutation that was conveyed to her daughter with autosomal dominant pattern. Knowing the form of inheritance, we proceeded to inform the patient and make genetic counseling, including discussion of potential risks and potential reproductive choices.

The daughter (Case 2) inquired as to how to have a child free of this disease. Because the mutation that causes the OI has identified there are 3 reproductive options: PGD, following treatment of *in vitro* fertilization, which allows genetically tested embryos and select non affections of OI to be transferred to uterus; another option would be an IVF treatment with donor eggs. This technique avoids the gestation of a child with OI, because it replaces the parent's diseased affected gamete by an anonymous healthy gamete. Another option would be to conceive a child and prenatal diagnosis after obtaining fetal cells by chorion biopsy or genetic amniocentesis, on which can be directed genetic studies of OI (only when the responsible disease mutation is already known in the family, which will be searched for in the fetus)[10].

The treatment of choice in adult patients with OI is not clearly established, but several studies have demonstrated the efficacy of both oral bisphosphonates as intravenous[11,12]. The usefulness of other drugs such as denosumab[13] and parathyroid hormone[14] has yet to be recognized.

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What are microRNAs? Potential biomarkers and therapeutic targets in osteoporosis

DOI: http://dx.doi.org/10.4321/S1889-836X2016000100007

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Date of receipt: 17/07/2015
Date of acceptance: 17/11/2015

Summary
Micro-RNAs (miRs) are small non-coding RNA molecules that regulate gene expression at post-transcriptional level. Generally, they act on gene expression by silencing or degrading mRNAs, and are involved in regulating various biological processes, such as cell differentiation, proliferation, apoptosis and in embryonic and tissue development. They are currently a major focus of interest in the study of various diseases such as cancer or type 2 diabetes mellitus. At level of bone metabolism, various miRs are emerging that are involved in their regulation, opening an important research field to identify new biomarkers for diagnosis of osteoporosis and its development, and to design new drug therapies.

Key words: *epigenetics, micro-RNA, biomarkers and osteoporosis.*

Introduction

MicroRNAs were discovered in 1993 to study the regulation of the development of the nematode *Caenorhabditis elegans*[1]. They are small noncoding RNAs (21-23 nucleotides) for proteins that constitute a large family of post-transcriptional regulatory genes. They are involved in the regulation of various biological processes such as cell differentiation, proliferation, and apoptosis in embryonic and tissue development[2]. They function as an epigenetic control of an endogenous gene and, although they generally silence genes which differ from those which have been transcribed, there are also microRNAs that promote and / or co-activate other genes[3].

Since their discovery, they have become one of the most studied in the field of epigenetic regulation of cells. Much of the current information has enhanced our understanding of the biological processes in which they are involved. Everything that has been carried out recently is a focus of interest for medicine as therapeutic targets in many diseases. To date, over 2,000 different human sequences of microRNAs have been described in the miRBase database (http://www.mirbase.org).

MicroRNAs represent only 2-3% of the human genome, and it is estimated they can regulate the expression of about 60% of genes[4]. One single miR can regulate about 200 different transcripts may act each in a different cellular pathway, and the same mRNA may be regulated by multiple microRNAs[5,6].

The genesis of microRNAs has been well studied and characterized by various authors[7-9]. First, inside the nucleus, the genes that encode for microRNAs are transcribed into long precursors, resulting in so-called primary microRNAs, varying in length from hundreds of base pairs. This precursor is cut by the Drosha and Pasha/DGCR8 ribonucleases in one or more RNA forked molecules, making it into pre-miRNAs of 60-70 nucleotides. The pre-miRNAs leave the nucleus and move toward the cytoplasm aided by exportin-5 where the miR maturation process takes place. In the cytoplasm, the pre-miRNA is carried by the RLC complex (RISC loading complex) formed by the RNase Dicer, TRBP (RNA binding protein in response to transactivation) prkra (activating RNA dependent protein kinase) and Ago2. This complex produces the cleavage of pre-miR generating a duplex with a mature miR chain and its complementary. The mature chain with Ago2 form the RISC complex (RNA-induced silencing complex) and the complementary strand will be removed. RISC binds to an mRNA molecule (usually in the 3 'untranslated) having a sequence complementary to the miR component and cuts the mRNA, leading to degradation of the mRNA or to modify its translation. Some microRNAs also serve as guides for the methylation of complementary sequences; both processes affect transcription. Biogenesis and mechanism by which they regulate microRNAs expression as shown in Figure 1.

The complementary sequence between mRNA and miR is only 7 nucleotides, it is believed that each miR could potentially mate with hundreds of different mRNAs. Similarly, a single RNA molecule could have multiple microRNAs binding sites. The translational inhibition binding must require several RISC complex to the same ARNm molecule[10].

MicroRNAs as biomarkers

Osteoporosis is a disease characterized by low bone mass and a deterioration in its quality, with weakening of the microarchitecture, leading to increased risk of fractures with minimal trauma. Although we now have several tools for assessing the risk of osteoporotic fractures, many low-risk patients suffer fractures and vice versa. The microRNAs could provide information to improve risk prediction. One of the challenges in the field of osteoporosis is early disease detection, allowing timely action and obtaining better results in treatment. This requires developing more effective noninvasive methods that are predictors of bone loss, fracture risk and/or therapeutic response, and allow us to monitor and assess the effectiveness of drug therapy.

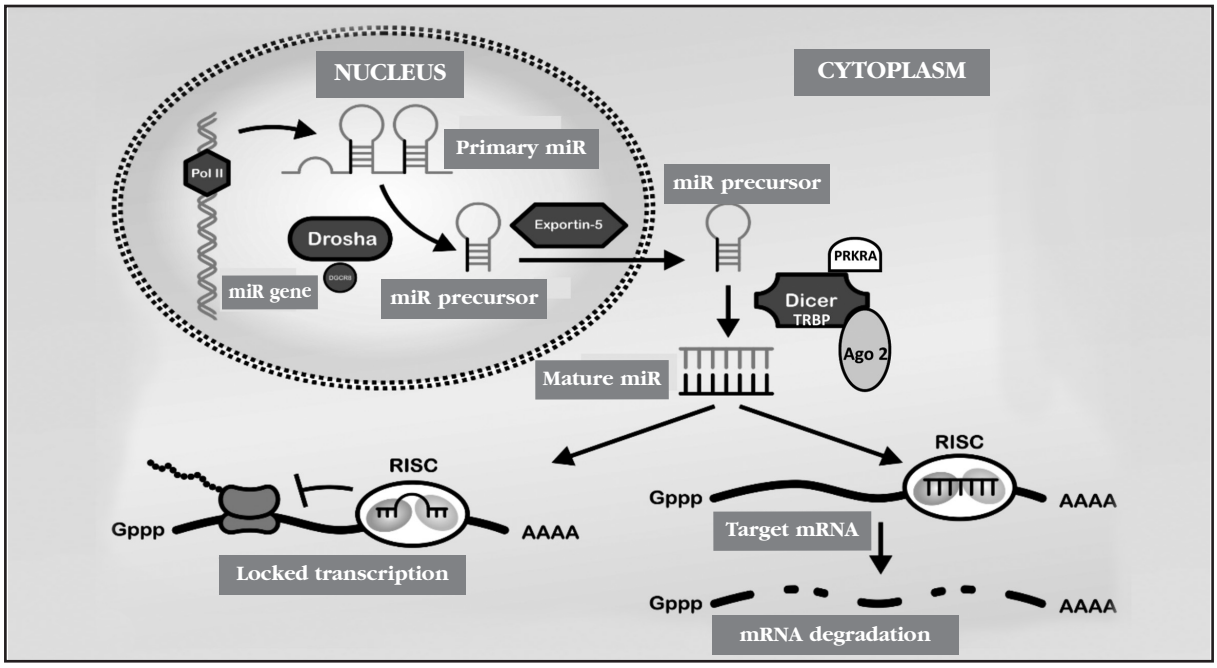
In this regard, the microRNAs may constitute new biomarkers of great interest, since they have been shown to resist RNase activity in peripheral blood, which affords them high stability in serum and plasma. On the other hand, they are reproducible and have high tissue specificity between individuals[11,12]. We know that the levels of expression of several microRNAs vary with aging, and a specific miR can have a positive and negative effect on the same cell depending on its state of differentiation[13]. Moreover, various alterations have already been described in the expression of microRNAs strongly related to the occurrence and development of diseases such as cancer, type 2 diabetes mellitus, Alzheimer's, osteoarthritis, among others[14,15]. Their quantification is already being used as biomarkers in the diagnosis and progression of these diseases[16-18].

In recent years, different microRNAs have begun to be described related to osteoporotic disease and/or risk of fracture[14,19-20], although data are still scarce.

The miR-2861 was the first miR that attributed a clinical implication in human osteoporosis[21]. The miR-2861 as a target molecule has a histone deacetylase (HDAC5) which negatively regulates RunX2. Mutations at the encoding locus induced osteoporosis. Animals treated with miR-286 inhibitors have a low bone mass phenotype. Subsequently, other microRNAs may play an important role in regulating bone metabolism as the target having several genes encoding crucial transcription factors in bone remodeling. However, it is still not known which of them is related to a higher or lower rate of bone turnover and low bone mass. In figures 2a and 2b we see the different microRNAs acting at both cells as osteoclastic and osteoblastic line[22-28].

Among the various microRNAs described to date, the role of some of them stand out as poten-

Figure 1. MiRNA biogenesis



tial biomarkers of osteoporotic fracture risk in our population. Specifically, we found that miR-21, miR-23a, miR-24, miR-25, miR-100, miR-125b, miR-miR-328-3p 518f are over-expressed in serum of women with fracture osteoporotic, whereas the expression of miR-187 is reduced[1,13,29]. Other studies point to miR-133a and miR-194-5p as possible biomarkers associated with osteoporotic disease, showing that in the serum of postmenopausal women are higher levels of such microRNAs, and are also negatively correlated with spine BMD and femoral neck[20,30].

Although there is increasing knowledge of more microRNAs involved in bone metabolism and biological function and its mechanism of action, the prior mechanism by which miRs reach the bloodstream is still not known, and their role in blood is not entirely understood. Future studies will clarify both aspects help us to find new and better biomarkers and more judiciously select those already proposed.

We should also bear in mind that, for the proper use of microRNAs as biomarkers in clinical practice, establishing a standard sample collection process and normalization techniques of real-time PCR is required.

The microRNAs as therapeutic targets

The microRNAs play a fundamental role in the regulation of bone metabolism. Variations in gene expression can lead to alterations in bone remodeling and have adverse effects on the skeleton. All this opens a new window of possibilities for the development of new therapeutic strategies for the treatment of various bone diseases such as osteoporosis.

The pharmaceutical industry is currently investigating drug targets aimed at normalizing the tissue levels of specific microRNAs, silencing those which were over-expressed or increasing their levels in those with a deficit. The microRNAs can be silenced by molecules called anti-miRNAs (AMOs). These are synthetic antisense oligonucleotides which competitively inhibit interaction between the microRNAs and its target mRNA. The most widely used AMOs are 2'-O-methyl AMO, 2'-O-methoxyethyl AMO and the Locked Nucleic Acids (LNAs)[31]. On the other hand, microRNAs often work in groups to regulate the pathological processes, so that instead of designing different anti-microRNAs for equal treatment, microRNAs called "sponge" are being developed, which can set numerous microRNAs at once.

Conversely, if we want to restore decreased levels of miR, the strategy is to manage microRNAs mimetic (miR mimics), which are molecules of dsRNA chemically altered to mimic endogenous microRNAs. When introduced into cells, they are recognized by miR mimics machinery microRNAs biogenesis and processed as such.

MicroRNAs use as pharmacological agents is already accepted in some tumor and viral pathologies[31]. At present, there are different molecules with inhibitory pharmacological action of microRNAs being used in phase II and III for the treatment of hepatitis C, miR-1 (miravirsen)[32] and RG-101[33].

As for the field of bone metabolism, advances in therapy are lower. We can find only a few isolated studies working in cell or animal models. Notably, a recent study published in Nature in which the miR-34a is designated as a new suppressor of osteoclast formation and bone resorption, which has important implications for the treatment of osteoporosis or bone metastases. This study shows how mice with increased levels

Figure 2a. Targets and function of the miRNA on the differentiation and proliferation of osteoblasts

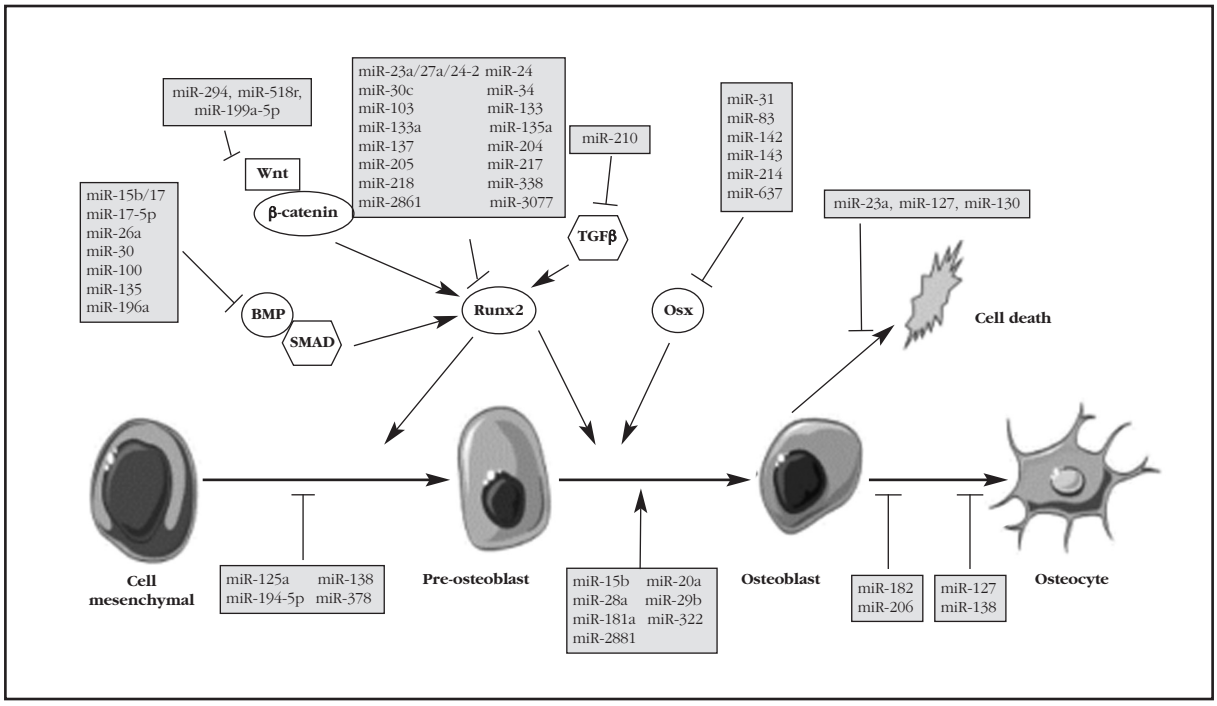
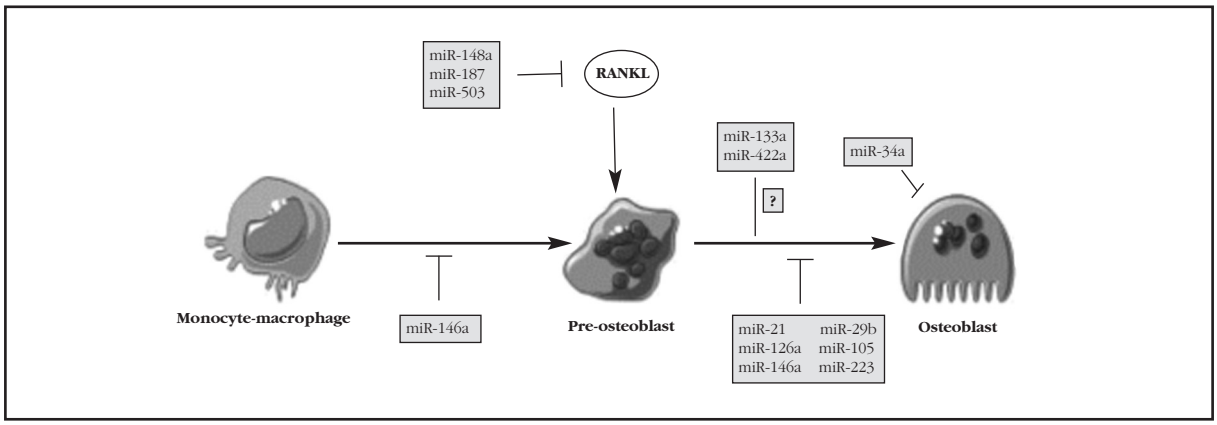


Figure 2b. Targets and function of the miRNA on the differentiation and proliferation of osteoblasts



of miR-34a have a higher bone density and lower rate of bone fractures. After injection of nanoparticles containing microRNA, both bone loss in mice with postmenopausal osteoporosis and bone metastasis in mouse models of reduced breast or skin cancer were reduced[19]. Wang et al. injected anti-miR-214 in mice and observed a lower loss of bone mass in treated animals[13]. Currently there are two major limitations to the use of microRNAs as pharmacological agents. The first is that one miR tends to have different target genes at a time and also can act as an inhibitor or promoter, depending on the target gene and the stage of cell differentiation time. This complexity explains the difficulties in predicting the spectrum of action and toxicity profiles associated with microRNAs therapy. To avoid this issue, recent research focuses on testing the stability of the microRNAs and direct its action to target tissues or cells. The second limitation is that the unmodified microRNAs may trigger nonspecific reactions of interferon in tissues. The presence of anti-miR or miR mimics modulates the stimulators of interferon gene expression, causing changes in the immune response.

Conclusions

1. The role of microRNAs in gene regulation is essential. They are involved in the regulation of various biological processes such as cell differentiation, proliferation and apoptosis in embryonic development and tissue.

2. The differential expression of microRNAs induces changes in most stages of skeletal development, so that the process of bone remodeling is also regulated by different microRNAs.

3. The study of the diverse differential expression profiles of microRNAs in bone metabolism disorders lead us to identify new biomarkers of osteoporotic disease and its development.

4. Given that microRNAs have a crucial role in bone tissue, better knowledge could lead us to set new therapeutic targets.

5. Better understanding of biogenesis microRNAs and their role in the pathogenic processes provide new tools for the diagnosis and prognosis of osteoporotic disease and new therapeutic targets.

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