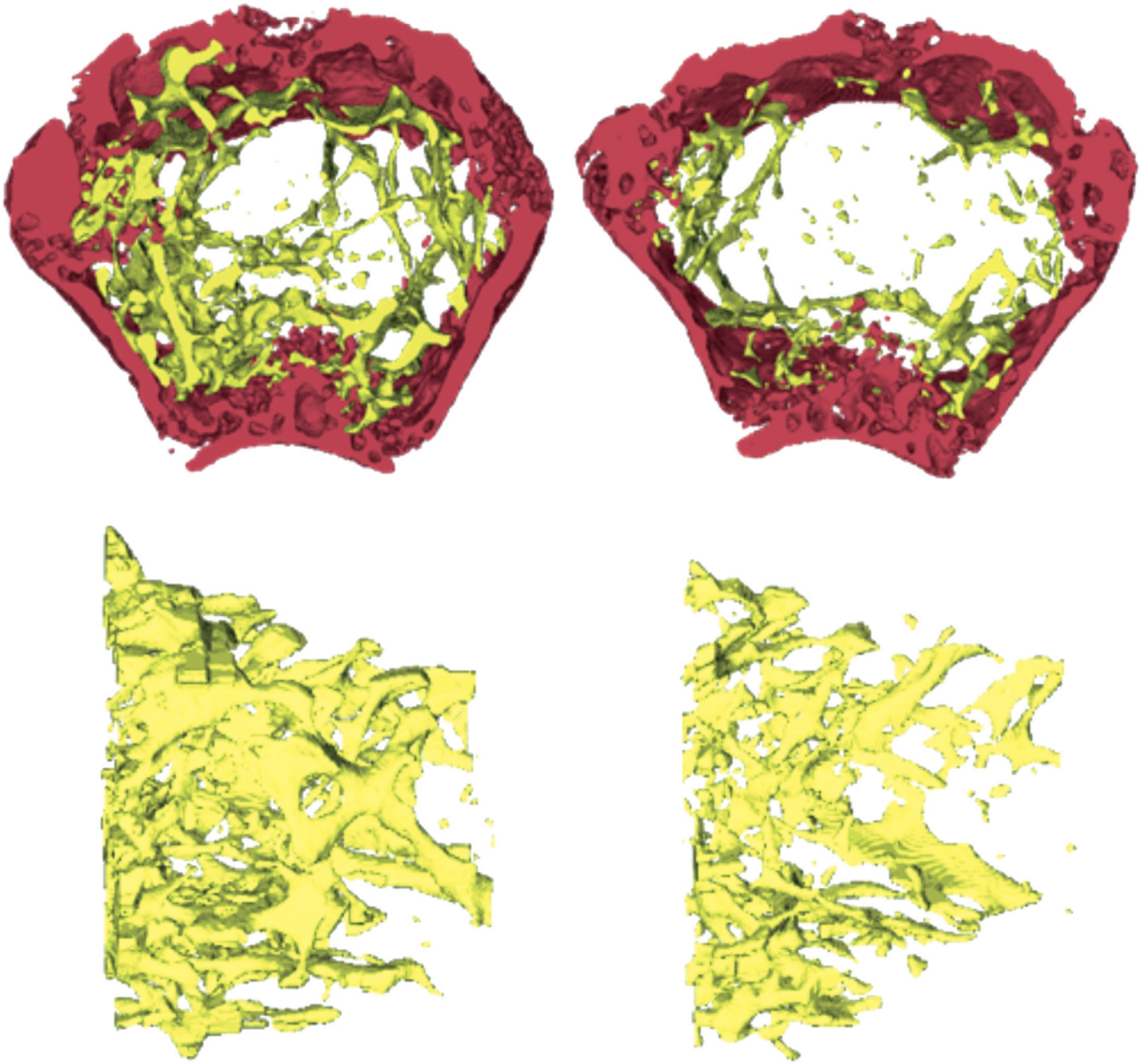
seiomm
Sociedad Española de Investigación Ósea y del Metabolismo Mineral

feiomm
Fundación Española de Investigación Ósea y del Metabolismo Mineral

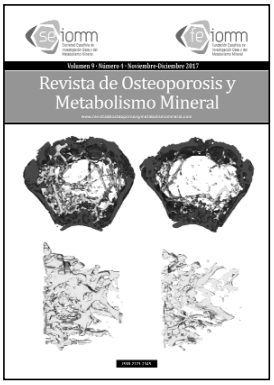
Volume 9 · Number 4 · November-December 2017

Revista de Osteoporosis y Metabolismo Mineral

www.revistadeosteoporosisymetabolismomineral.com



ISSN 2173-2345



Nuestra portada
Imágenes representativas de la microarquitectura 3D trabecular ósea obtenidas por microtomografía computarizada de secciones de fémur distal de rata: hueso no osteoporótico (izqda) *vs.* hueso osteoporótico

Autor:
Imágenes cedidas por el Dr. José R. Caeiro Rey, Trabeculae EBT S.L. San Cibrao das Viñas. Ourense

Velázquez, 94 (1ª planta)
28006 Madrid

Telf: +34-625 680 737
Fax: +34-917 817 020

e-mail: seiomm@seiomm.org

http://www.seiomm.org

Edición
ibáñez & Plaza Asociados, S. L.
EDITORIAL TÉCNICA Y COMUNICACIÓN
Avda. Reina Victoria, 47 (6º D)
28003 Madrid
Telf. +34-915 538 297
e-mail: correo@ibanezyplaza.com
http://www.ibanezyplaza.com

Maquetación
Concha García García

Traducción inglés
David Shea

ISSN: **2173-2345**

SUMARIO

Vol. 9 - Nº 4 - Noviembre-Diciembre 2017

Indexada en las siguientes bases de datos: Scielo, Web of Sciences, IBECS, SIIC Data Bases, embase, Redalyc, Emerging Sources Citation Index, 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.

Envío de originales: **romm@ibanezyplaza.com**

Revista de Osteoporosis y Metabolismo Mineral ha sido aceptada para su inclusión en *"Emerging Sources Citation Index"*, la nueva edición de *Web of Sciences* que funciona desde noviembre de 2015. Por ello, los artículos publicados en nuestra revista serán indexados en *Web of Sciences* desde el mismo momento de su publicación.

Editorial Committee

Reviewers Volume 9 (2017)

Diabetes and bone: an unexpected but intense relationship

DOI: http://dx.doi.org/10.4321/S1889-836X2017000400001

Jódar Gimeno E

Departamento de Endocrinología y Nutrición Clínica - Hospitales Universitarios Quirón Salud Madrid (Pozuelo, Ruber Juan Bravo, San José) - Madrid (España)
Facultad de Ciencias de la Salud - Universidad Europea de Madrid - Madrid (España)

e-mail: esteban.jodar@gmail.com

As a title of this editorial indicates, we have described the association between diabetes mellitus (DM) and osteoporosis as unexpected but intense. However, perhaps we should first say "still controversial for some." Today the proportion and intensity with which the disorders described in the quantity and quality of bone. In addition, alterations in metabolism are still under discussion. mineral, are associated with both type 1 diabetes (DMt1) and type 2 (DMt2), and influence an increase in the rate of fractures[1].

We know that diabetes juvenile onset may be associated with a reduction in the peak of bone mineral density (BMD), with the consequences that this entails for fractures at older ages. In addition, a greater than expected BMD has been described in DMt2, unlike DMt1, in which a reduction in BMD has been reported, especially associated with the appearance of chronic complications. In the current model, T2DM implies greater involvement of bone quality compared to reduced BMD, which would play a secondary role. In fact, there have been proposals to increase fracture risk estimates based on BMD (by multiplying them by up to 2)[2], due to the low predictive value of the former. Likewise, functional hypoparathyroidism has been described in people with DM, in addition to disorders secondary to the appearance of nephropathy or other chronic complications.

Finally, we cannot ignore the increased risk of falls secondary to neuropathy, visual disturbances, cerebrovascular disease or hypoglycemia itself, which cause many of the most classic treatments for diabetes, such as sulfonylureas and other secretagogues or insulin[1].

In addition, people with diabetes suffer an unexpectedly high number of fractures in the appendicular skeleton (arms, ankles, legs...), so a role for neuropathy, and even microangiopathy localized in these areas, has been suggested.

In this issue, Martínez-Laguna et al.[3] seek to determine if there are differences in the use of drugs between people with T2DM and without diabetes using the powerful database of Primary Care in Catalonia (Information System for the Development of Research in Primary Care, SIDIAP). When selecting subjects with DMt2 older than 50 years and matching them with two similar non-diabetic groups, the analysis of their clinical characteristics and treatments yielded very interesting data.

First, a fracture rate was corroborated –prevalence actually– much higher than expected –in fact, almost excessively high: 1.3% vs 0.3% in subjects without DM–, but what is even more troubling, even with this enormous prevalence of fractures (which we must assume as osteoporotic in the main), is that the use of, for example, bisphosphonates was 30% lower in people with diabetes, which is unacceptable.

The multivariate analysis encouraged to clear the role of confusing variables, confirmed that the diabetic sufferer had a lower probability of being treated for osteoporosis.

Therefore, in addition to welcoming this new interesting research by the group led by Daniel Prieto Alhambra, we also remember that there are updated recommendations for assessing osteoporosis secondary to endocrine diseases[2], and even specific recommendations on anti-diabetic treatments and their impact on fracture risk[4], promoted by the Working Group on Osteoporosis and Metabolic Bone Diseases of the Spanish Society of Endocrinology. We again recommend this work to all those concerned with treatment of osteoporosis and diabetes, a pair of conditions with a much more intense relationship than expected.

Bibliography

1. Napoli N, Chandran M, Pierroz DD, Abrahamsen B, Schwartz AV, Ferrari SL. Mechanisms of diabetes mellitus- induced bone fragility. Nat Rev Endocrinol. 2017;13(4):208-19.
2. Reyes-García R, García-Martín A, Varsavsky M, Rozas-Moreno P, Cortés-Berdonces M, Luque-Fernández I, et al. Actualizacioń de las recomendaciones para la evaluacioń y tratamiento de la osteoporosis asociada a

enfermedades endocrinas y nutricionales. Grupo de Trabajo de Osteoporosis y Metabolismo Mineral de la SEEN. Endocrinol Nutr. 2015;62(5):e47-e56.
3. Martínez-Laguna D, Reyes C, Carbonell-Abella C, Losada Grande E, Soldevila Madorell B, Mauricio D, et al. Uso de fármacos para la osteoporosis en pacientes con diabetes mellitus tipo 2: estudio de cohortes de base poblacional. Rev Osteoporos Metab Miner. 2017;9(4):107-13.
4. Rozas-Moreno P, Reyes-García R, Jódar-Gimeno E, Varsavsky M, Luque-Fernández I, Cortés-Berdonces M, et al. Recommendations on the effect of antidiabetic drugs in bone. Endocrinol Diabetes Nutr. 2017;64 (Suppl 1):1-6.

Martínez-Laguna D[1,2,3], Reyes C[2,3], Carbonell-Abella C[1,2,3], Losada Grande E[4], Soldevila Madorell B[5,6], Mauricio D[5,6], Díez-Pérez A[3,7], Nogués X[2,3,7], Prieto-Alhambra D[2,3,8]

1 Atención Primaria Barcelona Ciutat - Instituto Catalán de la Salud - Barcelona (España)

2 Grupo de Investigación en Enfermedades Prevalentes del Aparato Locomotor en Atención Primaria (GREMPAL) - Instituto Universitario de Investigación en Atención Primaria (IDIAP) Jordi Gol - Universidad Autónoma de Barcelona - Barcelona (España)

3 Área de Fragilidad y Envejecimiento Saludable del Centro de Investigación Biomédica en Red (CIBERFES) - Instituto de Salud Carlos III (ISCIII) - Madrid (España)

4 Unidad de Endocrinología - Hospital Can Misses - Ibiza (España)

5 Servicio de Endocrinología y Nutrición - Hospital Universitario Germans Trias i Pujol - Badalona (España)

6 Área de Diabetes y Enfermedades Metabólicas Asociadas del Centro de Investigación Biomédica en Red (CIBERDEM) - Instituto de Salud Carlos III (ISCIII) - Madrid (España)

7 Departamento Medicina Interna - Instituto de Investigaciones Médicas del Hospital del Mar (IMIM) - Universidad Autónoma de Barcelona - Barcelona (España)

8 Departamento de Ortopedia, Reumatología y Ciencias Musculoesqueléticas de Nuffield (NDORMS) - Unidad de Investigación Biomédica Músculoesquelética del Instituto Nacional para la Investigación en Salud (NIHR) - Universidad de Oxford (Reino Unido)

Use of drugs for osteoporosis treatment in patients with type 2 diabetes mellitus: population-based cohort study

DOI: http://dx.doi.org/10.4321/S1889-836X2017000400002

Correspondence: Daniel Prieto-Alhambra - Musculoskeletal Pharmaco and Device Epidemiology, Botnar Research Centre - Nuffield Orthopaedics Centre - Windmill Road - Oxford OX3 7LD (United Kingdom)
e-mail: Daniel.prietoalhambra@ndorms.ox.ac.uk

Date of receipt: 20/03/2017
Date of acceptance: 21/05/2017

Work awarded with a Clinical Research Fellowship FEIOMM 2014.

Summary

Objective: Ascertain whether there are differences in the prevalence of osteoporosis drugs in patients with type 2 diabetes (DM2) and non-diabetic patients.

Material and methods: Retrospective cohort study with data from the Information System for the Development of Primary Care Research (SIDIAP), which contains anonymous clinical information from more than 5 million patients in Catalonia. All 50-year-old patients diagnosed with DM2, who were matched with two subjects without diabetes, were selected. Information on descriptive variables, prevalent fractures and the use of osteoporosis drugs grouped in bisphosphonates (BF), calcium and vitamin D supplements (CaD), and any osteoporosis drug (OD) were collected. Through logistic regression, the association between the presence of DM2 and the use of OD was calculated, adjusting for confounding variables.

Results: A total of 166,106 patients with DM2 and 332,212 non-diabetics. The DM2 group presented a higher prevalence of fracture than did diabetics (1.3% vs 0.3%). The use of BF in patients with DM2 was 6.6%, compared to 9.3% in non-diabetics ($p<0.001$). Of CaD, 9.7% vs 12.3% ($p<0.001$) and OD, 7.6% vs 10.7% ($p<0.001$). After adjusting for variable confounders, the patients with DM2 presented a lower probability of being treated with BF (OR=0.67, 95% CI: 0.64-0.68), with CaD (OR=0.71, 95% CI: 0.70-0.73) or with OD (OR=0.66, 95% CI: 0.64-0.67) than non-diabetics.

Conclusions: Despite having a higher prevalence of fractures in patients with DM2, they have more than 30% chance of not having received an OD than non-diabetic patients. This may be attributed to an underestimation of risk in these patients..

Key words: *osteoporosis, bisphosphonates, type 2 diabetes mellitus, epidemiology, general population studies.*

Introduction

Osteoporosis is a disease of bone metabolism characterized by increased bone fragility and fracture propensity. In postmenopausal women, these fractures have been associated with a decrease in bone mineral density (BMD)[1]. However, this correlation with low BMD does not occur in all situations; Patients with type 2 diabetes mellitus (DM2) present an increased risk of fractures, especially femoral fractures[2-4], despite presenting higher BMD values compared to the non-diabetic population that fracture[5,6].

Different mechanisms have been postulated through which the risk of fracture in the diabetic population could be increased. These include some complications associated with DM2 (hypoglycemia, neuropathy, nephropathy and diabetic retinopathy)[7-10] and also associated with an increased risk of falls and, consequently, fractures. Also, some antidiabetic drugs, such as sulfonylureas, glitazones and insulin, have been associated with an increased risk of fractures[11,12]. An increased risk of fractures has also recently been reported in patients treated with a sodium 2-glucose co-transporter inhibitor type 2 (iSGLT-2), canagliflozin. This has not been observed so far with other iSGLT-2[13,14]. Another possible explanation would be the effect of deposition of advanced glycosylation products on bone collagen that may decrease bone strength[15-18].

Different osteoporosis drugs (OD) are available for the prevention of osteoporotic fractures. These have been analyzed in a multitude of clinical trials, varying their effect depending on the drug, the population studied and the location of the fracture. However, there is little information on these drugs in normal clinical practice, especially in diabetic patients[19-22].

If patients with T2DM have a higher BMD than non-diabetics and an increased risk of bills, it seems logical to think that the assessment of the real risk of fractures in these patients is underestimated and, consequently, under-treated. Our objective was to determine if there were differences in the prevalence of osteoporosis drugs among patients with DM2 and non-diabetic patients.

Material and methods

Study design:

Population-based retrospective cohort study with data from the Information System for the Development of Primary Care Research (SIDIAP) (www.sidiap.org). The SIDIAP contains the sociodemographic information, clinical records of primary care physicians working at the Catalan Institute of Health (ICS), the main provider of health services in Catalonia, as well as analytical results and pharmacy billing data. It has information of more than 5 million patients (approximately 80% of the Catalan population). The representativeness of SIDIAP over the general population of Catalonia has been previously demonstrated[23]. Previous studies carried out with SIDIAP in patients with DM2 observed a prevalence of the disease similar to studies done in other parts of Spain[24,25]. Various studies are also available that analyze new predictors of fragility fracture[26-30].

Participants:

There were selected all the subjects of 50 or more years of age by diagnosis of DM2 prevalent or incident between 2006 and 2013, using codes CIE10 (E11.0, E11.1, E11.2, E11.3, E11.4, E11.5, E11.6, E11.7, E11.8 and E11.9). For every person with DM2, two nondiabetic subjects were selected of the same sex, age (± 2 years) and from the same health center. Those subjects with no diagnosed DM2 or type 1 were considered non diabetic and not to receive any anti-diabetic medication before being included.

Study variables:

Data on age, sex and some clinical variables were collected: body mass index (BMI), smoking (smoker, non-smoker and former smoker) and alcohol consumption (measured by units consumed per week and classified as: low-risk consumption, when consumption in men is less than 17 units or in women to 11, moderate consumption, when in men is between 17 and 28 units or in women between 11 and 17, and consumption of risk when in men is Higher than 28 units or in women at 17, as defined in the Program of Preventive Activities and Health Promotion)[31]. The presence of ischemic heart disease (stable angina, unstable angina or myocardial infarction) and cerebrovascular disease (cerebral infarction or transient ischemic attack) were evaluated at the time of inclusion, using CIE10 codes. Prevalent fractures were also collected (from any location except face or skull, and fingers or toes). The use of drugs for osteoporosis was grouped into three categories: 1) bisphosphonates (BF), 2) supplements calcium and vitamin D (CaD), and 3) any osteoporosis drug (OD). The Anatomical Therapeutic Chemical Classification (ATC) codes were used for this purpose.

Statistical analysis:

The characteristics of the studied population are described by uni-variate descriptive analysis, calculating the mean and standard deviation for the continuous variables, and the absolute frequency and percentage for the categorical variables. Chi square test was used to compare the prevalence of cardiovascular disease and fractures in both groups. The association between the presence of DM2 and the use of OD was calculated through logistic regression; Was adjusted for the following confounding factors, defined a priori according to available literature and biological plausibility: age, sex, BMI, smoking, alcohol consumption, ischemic heart disease (ICH) or previous cerebrovascular disease (CVD) and previous fractures. All statistical tests were performed with a 95% confidence interval (CI) and assuming a bilateral contrast. The statistical package Stata SE version 12.0 for Mac was used for all analyzes.

Ethical considerations:

SIDIAP provided wholly observational data for this study. The SIDIAP data are totally anonymous and identified by an internal code created at the

moment of data inclusion, so it is impossible to identify the subjects included. Approval was obtained from the local Clinical Research Ethics Committee (CEIC IDIAP Jordi Gol), code P15/150.

Results

We identified 166,106 patients diagnosed with DM2 prevalent or incident between 2006 and 2013, and were matched with 332,212 non-diabetic patients. The baseline characteristics of both cohorts are shown in Table 1. Subjects with DM2 had a higher prevalence of IHD and CVD than non-diabetics. They also had a higher prevalence of previous fractures, in general and by specific locations (Table 2).

Patients with DM2 presented a lower proportion of drug use for osteoporosis, both BF and any OD and also for CaD, statistically significant ($p<0.001$ in all three situations) compared to non-diabetic patients (Figure 1).

When analyzing the likelihood of receiving a drug for osteoporosis in subjects with T2DM, compared to non-diabetic subjects, the unadjusted odds ratios were 0.67 (95% CI: 0.65-0.68) for BF, from 0.74 (95% CI: 0.72-0.75) for CaD, and 0.66 (95% CI: 0.65-0.68) for any OD.

After adjusting for age, sex, BMI, smoking, alcohol consumption, previous IC or CVD and previous fractures, subjects with DM2 were less likely to be treated with BF (OR=0.67; 95% CI: 0.64-0.68), with CaD (OR=0.71, 95% CI: 0.70-0.73) or with any OD (OR=0.66, 95% CI: 0.64-0, 67) than non-diabetics.

Discussion

Patients with DM2, despite having a higher prevalence of previous fractures, had more than a 30% probability of not receiving a drug for osteoporosis, compared to non-diabetic subjects. As in previous studies, we observed a higher proportion of fractures in patients with T2DM compared to non-diabetic patients, especially at the femur level, where the prevalence was multiplied by four. These data coincide with two recent meta-analyzes where 30% more risk of femur fracture is described in patients with DM2[3,32].

Paradoxically, even with a higher prevalence of previous fractures, patients with DM2 are less likely to be treated with a bisphosphonate, calcium and vitamin D supplements, or with any osteoporosis drug. One possible explanation for these events could be an underestimation of the risk of fracture in these subjects. Although we do not have data in our BMD cohort, previous studies comparing patients with T2DM with non-diabetic patients observed that the former had a higher BMD[5]. Therefore, if the fracture risk assessment is performed exclusively by BMD values, patients with DM2 would be undervalued. Another possibility would be the assessment of fracture risk through the use of tools that allow the calculation of the absolute risk of fracture.

In our area, the most commonly used tool is FRAX®, which does not consider DM2 a risk factor. Different studies[33,34] support the idea of not using FRAX® in patients with DM2, since at the same absolute risk value calculated by FRAX® patients with DM2 present a greater real risk of fractures than non-diabetic patients[35]. In an analysis of the Manitoba cohort, it was observed that patients with DM2 had a higher proportion of observed fractures than expected, both main and femoral fractures, a fact that did not occur in non-diabetic subjects[36]. A third plausible explanation would be that patients with DM2 receive more drugs than non-diabetics, and this could condition the clinician when prescribing a drug for osteoporosis. Although we do not have the number of drugs that our patients received on average, other studies carried out on patients with DM2 from the SIDIAP database[37] describe medication costing almost double compared to non-diabetic patients and, consequently, a greater number of drugs.

As expected, the DM2 patients in our cohort had a higher prevalence of IHD and CVD than nondiabetic patients, almost double. Some authors suggest that there is a relationship between cardiovascular disease and bone metabolism. A case-control study in subjects with metabolic syndrome observed that patients with a coronary event in the last six months had a higher prevalence of vertebral fracture and of any location compared to subjects who had not had a coronary event[38].

One of the limitations of our study is that the data come from the computerized medical history and, unlike the classic cohort studies, there is no case-by-case validation of each fracture. Previous studies have validated SIDIAP data compared to classic cohort studies and hospital discharge databases, with a moderate sensitivity (close to 70%) and high specificity (>95%)[39]. In addition, the ICD-10 coding does not distinguish between trauma fractures and fragility fractures. A recent validation of a sample of more than 300 fractures recorded in patients >50 years of age on the basis of SIDIAP found that more than 90% of femur fractures, more than 87% of vertebral fractures and more than 80% of fractures The main ones were due to fragility (not related to trauma)[40], which gives greater validity to our data. Another possible limitation is that the data in relation to the prescription are collected from the billing data to Pharmacy, in such a way that there may be a stated prescription not withdrawn at the pharmacy and, therefore, not considered. But this fact would occur in both cases in both DM2 and non-diabetic patients.

In contrast, this study has important strengths such as the high number of individuals included, which allows for the detection of statistically significant differences that in other cohort studies with a smaller sample size would not have been detected.

We consider necessary the search for tools that provide a better estimate of the risk of fractures in patients with DM2. One possibility could be to incorporate DM2 as a risk factor in FRAX® or to have a specific tool for patients with DM2 that takes into account both classic and DM2 risk factors. Another option would be the incorporation

Table 1. Baseline characteristics in paired T2D and non-diabetic patients

Variable	Patients with DM2 (n=166,106)	Non-diabetic patients (n=332,212)	Value of p
Gender ♀; n (%)	79,249 (47.7)	158,498 (47.7)	1
Age; mean ± SD	65,4 ± 11.4	63,8 ± 11.8	<0.001
BMI (kg/m²); n (%)			<0.001
<24.99	17,076 (10.3)	55,088 (16.6)	
25-29.99	60,404 (36.4)	112,913 (34.0)	
>30	75,923 (45.7)	79,220 (23.8)	
Losses	12,703 (7.6)	84,991 (25.6)	
Smoking; n (%)			<0.001
Non smoker	78,593 (47.3)	142,888 (43.0)	
Smoker	23,821 (14.4)	42,736 (12.9)	
Former smoker	16,835 (10.1)	26,137 (7.9)	
Losses	45,857 (28.2)	120,451 (36.2)	
Consumption of alcohol; n (%)			<0.001
Teetotaler	100,203 (60.3)	164,381 (49.5)	
Low risk	42,167 (25.4)	81,081 (24.4)	
Harmful consumption	5,257 (3.2)	8,924 (2.7)	
Losses	18,479 (11.1)	77,826 (23.4)	
CVD previous; n (%)	9,762 (5.9)	10,039 (3.0)	<0.001
IHD previous; n (%)	16,416 (9.9)	13,678 (4.1)	<0.001

♀: women; SD: standard deviation; BMI: body mass index; CVD: cerebrovascular disease; IHD: ischemic heart disease.

Table 2. Prevalence of fractures in patients with T2DM and non-diabetic pairs

Localization	Patients with DM2 (n=166,106)	Non-diabetic patients (n=332,212)	Value of p
Any localization n (%)	4,012 (2.4)	1,732 (0.5)	<0.001
Main fracture* n (%)	2,215 (1.3)	1,055 (0.3)	<0.001
Femur n (%)	609 (0.4)	382 (0.1)	<0.001

* Fracture of hip, wrist, forearm, humerus or vertebral.

of new techniques, such as micro-indentation, which allow the assessment of fracture risk independently of BMD[41,42].

Conclusions

Patients with DM2 are about 30% more likely to not receive bisphosphonate, calcium and vitamin D supplements or any osteoporosis drug than non-diabetic patients. We believe that this lower probability of being treated is due to an underestimation of the real risk of fracture in patients with T2DM, which justifies the need for a specific tool for the estimation of fracture risk in these patients.

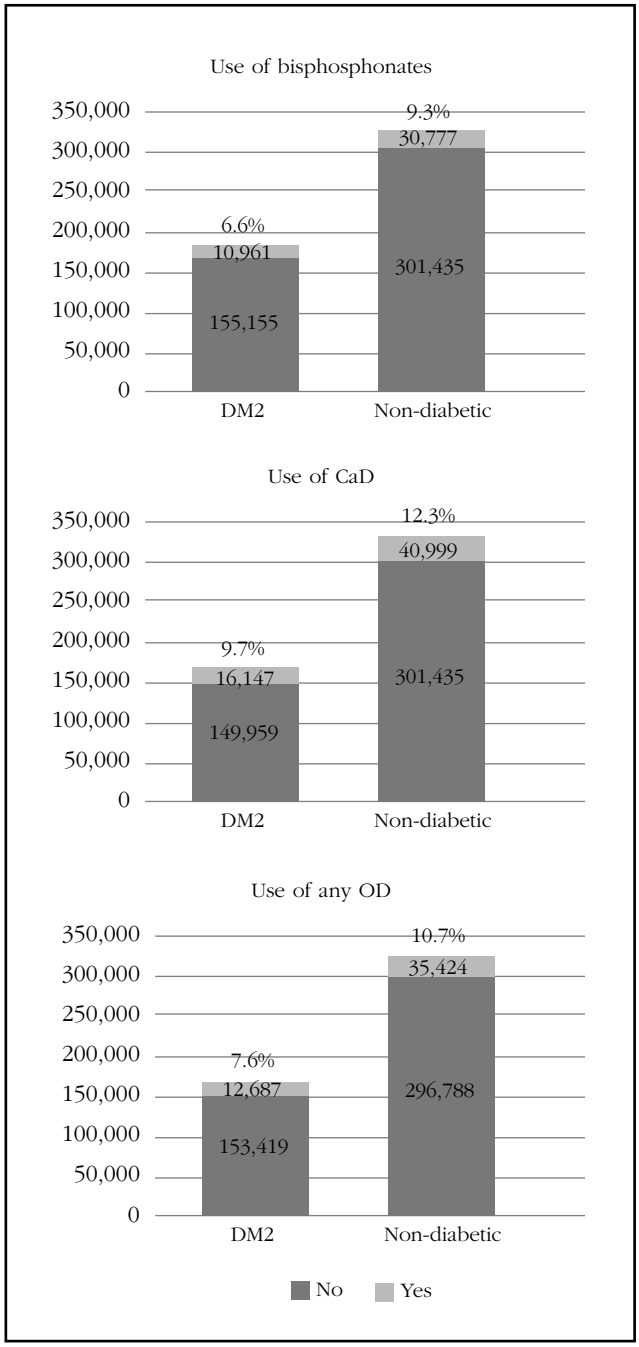
Funding: This paper was supported by the FEIOMM 2014 Clinical Research Grant and received an Italfarmaco award for the best Oral Communication of the Congress of SEIOMM 2016 held in Gran Canaria, Spain.

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

Bibliography

1. Siris ES, Miller PD, Barrett-Connor E, Faulkner KG, Wehren LE, Abbott TA, et al. Identification and fracture outcomes of undiagnosed low bone mineral density in postmenopausal women: results from the National Osteoporosis Risk Assessment. JAMA. 2001;286:2815-22.
2. Janghorbani M, Van Dam RM, Willett WC, Hu FB. Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. Am J Epidemiol. 2007;166:495-505.
3. Dytfeld J, Michalak M. Type 2 diabetes and risk of low-energy fractures in postmenopausal women: meta-analysis of observational studies. Aging Clin Exp Res. 2017;29(2):301-9.
4. Fan Y, Wei F, Lang Y, Liu Y. Diabetes mellitus and risk of hip fractures: a meta-analysis. Osteoporos Int. 2016;27:219-28.
5. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes--a meta-analysis. Osteoporos Int. 2007;18:427-44.
6. Ma L, Oei L, Jiang L, Estrada K, Chen H, Wang Z, et al. Association between bone mineral density and type 2 diabetes mellitus: A meta-Analysis of observational studies. Eur J Epidemiol. 2012;27: 319-32.
7. Johnston SS, Conner C, Aagren M, Ruiz K, Bouchard J. Association between hypoglycaemic events and fall-related fractures in Medicare-covered patients with type 2 diabetes. Diabetes Obes Metab. 2012;14:634-43.
8. Barzilay JI, Bůžková P, Chen Z, de Boer IH, Carbone L, Rassouli NN, et al. Albuminuria is associated with hip fracture risk in older adults: the cardiovascular health study. Osteoporos Int. 2013;24:2993-3000.
9. Ivers RQ, Cumming RG, Mitchell P, Peduto AJ. Diabetes and risk of fracture: The Blue Mountains Eye Study. Diabetes Care. 2001;24: 1198-203.
10. Rasul S, Ilhan A, Wagner L, Luger A, Kautzky-Willer A. Diabetic polyneuropathy relates to bone metabolism and markers of bone turnover in elderly patients with type 2 diabetes: greater effects in male patients. Gend Med. 2012;9:187-96.
11. Zhu Z-N, Jiang Y-F, Ding T. Risk of fracture with thiazolidinediones: An updated meta-analysis of randomized clinical trials. Bone. 2014;68:115-23.
12. Majumdar SR, Josse RG, Lin M, Eurich DT. Does sitagliptin affect the rate of osteoporotic fractures in type 2 diabetes? population-based cohort study. J Clin Endocrinol Metab. 2016;101:1963-9.
13. Watts NB, Bilezikian JP, Usiskin K, Edwards R, Desai M, Law G, et al. Effects of canagliflozin on fracture risk in patients with type 2 diabetes mellitus. J Clin Endocrinol Metab. 2016;101:157-66.
14. Ljunggren Ö, Bolinder J, Johansson L, Wilding J, Langkilde AM, Sjöström CD, et al. Dapagliflozin has no effect on markers of bone formation and resorption or bone mineral density in patients with inadequately controlled type 2 diabetes mellitus on metformin. Diabetes Obes Metab. 2012;14:990-9.
15. Miyata T, Notoya K, Yoshida K, Horie K, Maeda K, Kurokawa K, et al. Advanced glycation end products enhance osteoclast-induced bone resorption in cultured mouse unfractionated bone cells and in rats implanted subcutaneously with devitalized bone particles. J Am Soc Nephrol. 1997;8:260-70.
16. Jehle PM, Jehle DR, Mohan S, Böhm BO. Serum levels of insulin-like growth factor system components and relationship to bone metabolism in Type 1 and Type 2 diabetes mellitus patients. J Endocrinol. 1998;159:297-306.
17. Takagi M, Kasayama S, Yamamoto T, Motomura T, Hashimoto K, Yamamoto H, et al. Advanced glycation endproducts stimulate interleukin-6 production by human bone-derived cells. J Bone Miner Res. 1997;12:439-46.
18. Yamamoto M, Yamaguchi T, Yamauchi M, Yano S, Sugimoto T. Serum pentosidine levels are positively associated with the presence of vertebral fractures in postmenopausal women with type 2 diabetes. J Clin Endocrinol Metab. 2008;93:1013-9.
19. Vestergaard P, Rejnmark L, Mosekilde L. Are antiresorptive drugs effective against fractures in patients with diabetes? Calcif Tissue Int. 2011;88:209-14.
20. Johnell O, Kanis JA, Black DM, Balogh A, Poor G, Sarkar S, et al. Associations between baseline risk factors and vertebral fracture risk in the Multiple Outcomes of Raloxifene Evaluation (MORE) Study. J Bone Miner Res. 2004;19:764-72.
21. Ensrud KE, Stock JL, Barrett-Connor E, Grady D, Mosca L, Khaw K-T, et al. Effects of raloxifene on fracture risk in postmenopausal women: the Raloxifene Use for the

Figure 1. Use of osteoporotic drugs based on the presence or not of DM2



Heart Trial. J Bone Miner Res. 2008;23:112-20.
22. Schwartz AV, Pavo I, Alam J, Disch DP, Schuster D, Harris JM, et al. Teriparatide in patients with osteoporosis and type 2 diabetes. Bone. 2016;91:152-8.
23. García-Gil MM, Hermosilla E, Prieto-Alhambra D, Fina F, Rosell M, Ramos R, et al. Construction and validation of a scoring system for the selection of high-quality data in a Spanish population primary care database (SIDIAP). Inform Prim Care. 2011;19:135-45.
24. Vinagre I, Mata-Cases M, Hermosilla E, Morros R, Fina F, Rosell M, et al. Control of glycemia and cardiovascular risk factors in patients with type 2 diabetes in primary care in Catalonia (Spain). Diabetes Care. 2012;35:774-9.
25. Mata-Cases M, Franch-Nadal J, Real J, Mauricio D. Glycaemic control and antidiabetic treatment trends in primary care centres in patients with type 2 diabetes mellitus during 2007-2013 in Catalonia: a population-based study. BMJ Open. 2016;6(10):e012463.
26. Prieto-Alhambra D, Premaor MO, Fina Avilés F, Hermosilla E, Martinez-Laguna D, Carbonell-Abella C, et al. The association between fracture and obesity is site-dependent: a population-based study in postmenopausal women. J Bone Miner Res. 2012;27:294-300.
27. Prieto-Alhambra D, Premaor MO, Avilés FF, Castro AS, Javaid MK, Nogués X, et al. Relationship between mortality and BMI after fracture: a population-based study of men and women aged ≥40 years. J Bone Miner Res. 2014;29:1737-44.
28. Reyes C, Pottegård A, Schwarz P, Javaid MK, Van Staa TP, Cooper C, et al. Real-Life and RCT Participants: Alendronate Users Versus FITs' Trial Eligibility Criterion. Calcif Tissue Int. 2016;99:243-9.
29. Reyes C, García-Gil M, Elorza JM, Fina-Avilés F, Mendez-Boo L, Hermosilla E, et al. Socioeconomic status and its association with the risk of developing hip fractures: A region-wide ecological study. Bone. 2015;73:127-31.
30. Güerri-Fernandez R, Vestergaard P, Carbonell C, Knobel H, Avilés FF, Castro AS, et al. HIV infection is strongly associated with hip fracture risk, independently of age, gender, and comorbidities: a population-based cohort study. J Bone Miner Res. 2013;28:1259-63.
31. Córdoba García R, Camarelles Guillem F, Muñoz Seco E, Gómez Puente JM, Ramírez Manent JI, José Arango JS, et al. Recomendaciones sobre el estilo de vida. Aten Primaria. 2016;48(Suppl 1):27-38.
32. Fan Y, Wei F, Lang Y, Liu Y. Diabetes mellitus and risk of hip fractures: a meta-analysis. Osteoporos Int. 2016;27:219-28.
33. Bridges MJ, Ruddick S a. Do FRAX/NOGG guidelines predict fractures in post-menopausal women with Type 2 diabetes? Diabet Med. 2012;29:555-6.
34. Carnevale V, Morano S, Fontana A, Annese MA, Fallarino M, Filardi T, et al. Assessment of fracture risk by the FRAX algorithm in men and women with and without type 2 diabetes mellitus: a cross-sectional study. Diabetes Metab Res Rev. 2014;30:313-22.
35. Schwartz A V, Vittinghoff E, Bauer DC, Hillier TA, Strotmeyer ES, Ensrud KE, et al. Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. JAMA. 2011;305:2184-92.
36. Giangregorio LM, Leslie WD, Lix LM, Johansson H, Oden A, McCloskey E, et al. FRAX underestimates fracture risk in patients with diabetes. J Bone Miner Res. 2012;27:301-8.
37. Mata-Cases M, Casajuana M, Franch-Nadal J, Casellas A, Castell C, Vinagre I, et al. Direct medical costs attributable to type 2 diabetes mellitus: a population-based study in Catalonia, Spain. Eur J Health Econ. 2016;17:1001-10.
38. Silva HC, Pinheiro MM, Genaro PS, Castro CHM, Monteiro CMC, Fonseca FAH, et al. Higher prevalence of morphometric vertebral fractures in patients with recent coronary events independently of BMD measurements. Bone. 2013;52:562-7.
39. Pagès-Castellà A, Carbonell-Abella C, Avilés FF, Alzamora M, Baena-Díez JM, Laguna DM, et al. Burden of osteoporotic fractures in primary health care in Catalonia (Spain): a population-based study. BMC Musculoskelet Disord. 2012;13:79.
40. Martinez-Laguna D, Soria-Castro A, Carbonell-Abella C, Orozco P, Estrada-Laza P, Nogues X, et al. P172 Validation of fragility fractures in primary care electronic medical records: a population-based study. Osteoporos Int. 2016;27:79-548.
41. Farr JN, Drake MT, Amin S, Melton LJ, McCready LK, Khosla S. In vivo assessment of bone quality in postmenopausal women with type 2 diabetes. J Bone Miner Res. 2014;29:787-95.
42. Nilsson AG, Sundh D, Johansson L, Nilsson M, Mellström D, Rudäng R, et al. Type 2 diabetes mellitus is associated with better bone microarchitecture but lower bone material strength and poorer physical function in elderly women: a population-based study. J Bone Miner Res 2017;32:1062-71.

Bedia Díaz G, Carrillo López N, Solache Berrocal G, Dusso A, Rodríguez I, Naves Díaz M, Cannata Andía JB, Román García P
Servicio de Metabolismo Óseo y Mineral - Instituto Reina Sofía de Investigación Nefrológica - Red de Investigación Renal (REDinREN) del Instituto de Salud Carlos III (ISCIII) - Universidad de Oviedo - Hospital Universitario Central de Asturias - Oviedo (España)

Hypomethylation of the PTH gene due to high dietary phosphorus: a possible aggravating of severe secondary hyperparathyroidism in chronic renal failure

DOI: http://dx.doi.org/10.4321/S1889-836X2017000400003

Correspondence: Manuel Naves Díaz - Servicio de Metabolismo Óseo y Mineral - Hospital Universitario Central de Asturias - Edificio FINBA, Planta primera F1.1 (Aula 14) - Avenida de Roma, s/n - 33011 Oviedo (Spain)
e-mail: manuel@hca.es

Date of receipt: 18/05/2017
Date of acceptance: 02/07/2017

Awarded work with a FEIOMM Translational Research Grant 2014.

Summary

Introduction: Hyperphosphataemia aggravates both parathyroid hyperplasia and PTH secretion in patients with chronic kidney disease (CKD). Hyperplasia is associated with decreases in calcium receptor expression (CaSR), vitamin D (VDR) and α-Klotho, inducing resistance of the parathyroid gland to respond both to treatment and to increases in FGF23. This study examined the possible epigenetic contributions of raised phosphorus to aggravate secondary hyperparathyroidism (SHPT) in patients with (CRD).
Material and methods: The degree of methylation was compared by pyrosequencing of bisulfite in CpG-rich sequences of the promoters in the CaSR, VDR, PTH and α-Klotho genes in parathyroid gland DNA from uremic rats fed a normal and high phosphorus diet.
Results: The diet rich in phosphorus increased PTH expression and caused a marked reduction in the degree of methylation in the promoter of the PTH gene. In contrast, the promoter regions of the CaSR, VDR and α-Klotho genes did not show significant differences in the percentage of methylation between the two groups of rats. Thus, it was not the determining mechanism for the decrease of the expression of these genes observed in the SHPT.
Conclusions: The epigenetic alterations induced by the phosphorus rich diet in SHPT, particularly the PTH gene hypomethylation, could contribute to the increases that occur in the synthesis and secretion of this hormone. The identification of the mechanisms involved would allow better treatments for SHPT to be designed in the early stages of CKD.

Key words: *DNA methylation, PTH, chronic kidney disease, parathyroid glands, hiperphosphataemia.*

Introduction

Secondary hyperparathyroidism (SHPT) is a common complication of chronic renal disease (CRD) characterized by hyperplasia of the parathyroid glands and increases in the synthesis and secretion of parathyroid hormone (PTH). Raised serum levels PTH cause alterations in bone remodeling and phospho-calcium homeostasis that increase both fracture propensity and vascular calcification, a process that aggravates the morbidity and mortality of patients with renal problems[1].

In the course of CRD, the most important stimuli for the development of SHPT are decreases in circulating levels of calcium, nutritional vitamin D and its hormonal form, calcitriol, as well as raised serum phosphorus, when levels are below the upper limit of the normal range[2].

It is noteworthy that the degree of parathyroid hyperplasia in CRD is also associated with a proportional decrease in the parathyroid expression of calcium and vitamin D receptors (CaSR and VDR)[3]. These reductions diminish the gland's ability to suppress both cell proliferation and PTH secretion rates in response to changes in circulating levels of calcium and vitamin D induced by treatment to correct hypocalcemia or Vitamin D deficiency. An additional aggravating factor to parathyroid dysfunction of CRD is the early decrease of the anti-aging molecule, α-Klotho, in the membrane of parathyroid cells[4]. This reduction leads to an ineffective suppression of PTH synthesis and secretion by the phosphaturic FGF23 hormone, since α-Klotho acts as a co-receptor bound for cellular signals of the FGF23 complex with its specific receptor FGFR[5,6].

We now know that, in addition to the defects in the transcriptional control of the PTH, CaSR and α-Klotho gene due to calcitriol deficiency, or to decreased levels of its receptor, VDR, in the parathyroid gland hyperplastic,[3,4] epigenetic modifications such as hypermethylation of the CaSR, VDR or α-Klotho genes in their promoter regions may also contribute to renal parathyroid dysfunction. Interest in the epigenetics of SHPT in CRD arose from the evidence of the critical role of hypermethylation of tumor suppressor genes in processes of exacerbated cell proliferation[7,8], as occurs in nodular SHPT. This form of SHPT is similar in its development to a benign endocrine tumor, with a very serious adverse impact in the progression of SHPT, renal and vascular damage, as well as in the survival of the renal patient who develops resistance to treatment[9,10].

In contrast, evidence shows the significant impact of mild increases in α-Klotho gene methylation induced by aging in the brain and by uremic toxins in the kidney, both in α-Klotho expression in the cell membrane and in its anti-oxidant and anti-inflammatory functions which have not been studied in the parathyroid gland[11,12].

Another important epigenetic modification for controlling SHPT is the global hypomethylation of the PTH gene, demonstrated exclusively in the parathyroid tissue[13]. Although the degree of global hypomethylation of the PTH gene is similar in glands with normal function and hyperfunctioning glands[13] this finding suggests that a differential methylation process of this gene in its promoter regions may contribute to the severity of SHPT.

As phosphorus retention by the diseased kidney is the major risk factor for directly exacerbating the degree of parathyroid hyperplasia, stabilizing the messenger RNA of PTH, secretion of PTH into the circulation, and increasing FGF23 in the CRD, by non-transcriptional mechanisms, this study aimed to assess the possible contribution of epigenetic alterations induced by elevations in serum phosphorus to the severity of parathyroid dysfunction in a murine model of CRD. To do this, we compared the degree of methylation of the promoters of the CaSR, VDR, Klotho and PTH genes in uremic rats fed diets with normal or high phosphorus content and their association with the severity of SHPT.

Material and methods

Experimental Study

For the study, 4-month-old male Wistar rats from the University of Oviedo animal lab were subjected to a nephrectomy (NX) of 7/8 consisting of the elimination of three quarters of the left kidney and total resection of the right kidney[14].

Immediately after nephrectomy, a group of uremic animals continued with the maintenance diet for rodents with normal (N) content in phosphorus (P) (0.6%, NX-NP group), while the other group of nephrectomized animals received a diet with high (E) phosphorus content (0.9%; NX-EP group) for 20 weeks.

At the time of sacrifice, carried out under CO_2 anesthesia and by exsanguination, serum was collected to determine general markers of CRD grade and alterations in bone and mineral metabolism and also parathyroid glands in each experimental group (14 glands of 7 rats per group) stored at -80°C until use.

Analysis of methylation of the promoters of the genes under study by bisulfite pyrosequencing

To extract genomic material from the rat parathyroid glands, the phenol-chloroform method was used. The DNA extracted from the parathyroid glands was treated with sodium bisulfite following EZ DNA Methylation-Gold™ Kit D5005" instructions (Zymo Research, Orange, USA). A specific polymerase chain reaction (PCR) was then performed with biotinylated primers followed by the pyro-sequencing protocol (PyroMark QUIAGEN® Q24), which consists of denaturing the double strands of the PCR products to obtain single chains, one of them labeled with biotin. The biotinylated strand was used as a template to bind the sequencing primer. The methylation pattern of the promoter region of the PTH, VDR, CaSR and Klotho genes between the initiation of transcription to the 5' end was analyzed with Pyromark 2.0.6 software using the pairs of primers indicated in table 1.

Statistic analysis
For the analysis of the results the statistical program SPSS 17.0 was used. For the quantitative variables analyzed we used Student's t. Statistically significant differences were considered when values of $p<0.05$.

Results

Biochemical data
Biochemical data from both experimental groups are presented in table 2. As expected, animals fed the high phosphorus diet (NX-EP) presented a greater impairment of renal function measured as serum urea and creatinine, regarding the values of these parameters in the group of uremic rats fed with the diet with normal phosphorus content (NX-NP).

Although no significant differences were found in serum calcium levels between the two experimental groups, the combination of lower renal function and high dietary phosphorus led to marked increases in circulating levels of phosphorus and FGF23 on the order of 2 and 3 times higher than the values of these parameters in uremic animals fed with normal phosphorus. Consequently, the degree of SHPT was also higher in uremic animals with high phosphorus in the diet, showing 40-fold higher serum levels of PTH.

Methylation of the promoter regions of the genes under study
Figure 1 shows the percentages of methylation of the CpG sites in the parathyroid DNA included in the promoter regions of the CaSR, VDR and Klotho genes. The low percentage of methylation, less than 5% in both experimental groups, prevents any comparison of possible differential epigenetic alterations attributable to the high phosphorus in the diet compared to a normal phosphorus intake.

In contrast to these genes that decrease with SHPT progression, Figure 2 shows that in the two CpGs of the study area of the PTH gene promoter, in the parathyroid glands of normal-phosphorus-fed uremic rats there was a percentage of methylated parathyroid DNA greater than 40%. More importantly, for the same baseline grade of renal damage and duration of uremia (20 weeks), elevated phosphorus in the diet was associated with a significant 80% decrease in methylation of that region of the PTH promoter. This decrease is in line with increases in PTH levels in the serum of these animals 40 times higher than serum PTH in animals with the same basal grade of uremia fed a diet with normal phosphorus.

Discussion

This study is the first to show a possible epigenetic association between CRD hyperphosphatemia and SHPT severity: hypomethylation of the PTH gene. In addition, our results corroborate the findings of other researchers that decreases in parathyroid content of CaSR and VDR cannot be attributed to an epigenetic process of silencing by hypermethylation of these genes, both critical for effective treatment of SHPT. Our findings also question the contribution of α-Klotho promoter hypermethylation in membrane α-Klotho declines that occur with SHPT progression.

In general, CpG island hypermethylation in promoter regions results in silencing the transcription of genes. Our results indicate a degree of methylation of less than 5% in CaSR and VDR, both of which regulate normal parathyroid function and the development of resistance to treatment. In addition, high dietary phosphorus, which led to significant increases in both the degree of renal damage and the SHPT of these nephrectomized animals, did not induce significant changes in the

Table 1. Pair of primers used in mutilation studies

Primer	Sequence	MR_f	Size	CpG
Klotho F1	TGGAAAGTTTAGAATGGGAGAAAG			
Klotho R1	CCCTTTACCTTCCAAAAACTAAT	51.3	121 pb	5
Klotho SQ	GGGAAAGTAGGTGTTTTATT			
CaR FW1	AGTTTGGGAATGGTTATAGTT			
CaR RV1	CTCCCTAAATCTCTCAAATCAACCTTTA	52.7	169 pb	8
CaR SQ1	TAGGTGGTTTGGGGG			
PTH RW1	GGATTTTGAGTTTTGGGTTAGTTTGAT			
PTH RV1	ACCTAAATTTCATATACAAACCTTTTACT	52.9	360 pb	2
PTH SQ1	ATTTGAAATTTTAGAGGAGTG			
VDR FW1	AGGAATGTTAGGTAGGAGAGA			
VDR RV1	CCTTAAAAACCCTACCTTATAAAAAACTCT	52.6	344 pb	6
VDR SQ1	GATATTATTAAAGATTGT			

MT_f: PCR annealing temperature; F1: direct primer; R1: reverse primer; SQ: sequencing primer.

degree of methylation of CaSR or VDR, as also demonstrated by other investigators in murine models[15]. These did not measure the degree of global methylation of these two genes in human parathyroid glands from normal subjects or with variable grade of SHPT and primary, in which the contribution of hyperphosphatemia was not the main objective of the epigenetic analysis[16,17].

Regarding the degree of methylation of the anti-aging gene α-Klotho, its methylation degree was also less than 10%, and no significant differences were observed in the methylation percentage of the CpGs induced by high phosphorus in the diet, at least in the area of the promoter studied in the parathyroid glands from both experimental groups of uremic rats. These findings are not surprising, since in cells of the distal renal tubule, which is where the Klotho gene is expressed predominantly in a normal kidney, there seems to be a mechanism that actively protects the α-Klotho promoter from the methylation of CpGs sequences[18]. In other tissues, with low expression of α-Klotho, as in brain, breast, stomach, colon, skeletal muscle or skin, there also appears to be a similar mechanism of protection of α-Klotho levels preventing their methylation. It is also important to note that several authors have observed that a low degree of methylation appears to be sufficient to cause significant differences in the degree of gene expression. In fact, King et al. have shown in the brain of aged monkeys that a small 0.4% increase in CpG island methylation led to 20% declines in gene expression, corroborating that the degree of methylation of CpGs sequences may be involved in downward regulation of the Klotho gene associated with aging[13].

Other authors have also reported small differences (from 1 to 4.5%) in the degree of renal methylation in nephrectomized mice[13], similar to the parathyroid methylation values obtained in this study (2-5%). However, high dietary phosphorus did not lead to significant increases in the degree of methylation of this gene in parathyroid tissue.

Undoubtedly, the most important finding of this study has been the identification, for the first time, of an association between hyperphosphatemia and a decrease in methylation of the PTH promoter in the 350 nucleotide sequence preceding the start of transcription. Although studies of almost two decades ago, using techniques that preceded the development of pyrosequencing, demonstrated a global hypomethylation of the PTH gene exclusive of parathyroid tissue, but without significant differences between glands with normal or hyperfunctioning glands, the result of this study adds a possible epigenetic modification to the known post-transcriptional mechanisms induced by high phosphorus to markedly increase the synthesis and secretion of PTH, such as the stabilization of messenger RNA of PTH or the induction of secretory pathways[19-21]. It is important to note that the significant hypomethylation of PTH gene induced by high phosphorus in the diet could contribute in part to the marked elevations in serum PTH levels in this murine model of advanced experimental renal disease. However, we can not rule out with these results that, in fact, the normal or low phosphorus of the diet is the cause of the greater methylation of the PTH gene during the 20 weeks of uremia studied in this paper. In fact, phosphorus restriction in the diet does not affect the intraglandular content of PTH, but the capacity of the parathyroid cell for secretion into the circulation[22].

An important limitation of this study is that the impact of the methylation of these two CpGs on the transcription of the PTH gene has not been considered. Therefore, we can only postulate a potential mechanism to be analyzed in greater depth in the future to identify a cause-effect relationship between hypomethylation development of the PTH gene and its molecular control mechanisms, which would allow the incorporation of new therapeutic strategies for SHPT control in CRD.

In conclusion, these findings suggest that the development of epigenetic alterations such as the significant hypomethylation of the PTH gene, in advanced stages of parathyroid gland dysfunction in experimental renal disease, could contribute to the increase of both synthesis and secretion of PTH. The design of studies to obtain conclusive evidence of the impact that hypomethylation has on the synthesis of PTH and that lead to the identification of the molecular mechanisms responsible for this epigenetic modification induced by the high phosphorus in the diet is the first step for designing innovative therapeutic strategies for the effective treatment of SHPT from early stages of CRD.

Acknowledgments: This work was supported by a FEIOMM 2014 grant for the promotion of traslational research. Agustín Fernández Fernández from the Epigenetic Laboratory of Oviedo, Spain provided suggestions for the manuscript. This work has also been partially financed with the support of the National Plan for R & D & I 2008-2011, State Plan for R & D & I 2013-2016, Carlos III Health Institute (ISCIII) - European Regional Development Fund 09/00415), Science, Technology and Innovation Plan 2013-2017 of the Principality of Asturias (GRUPIN14-028), Foundation for the Promotion in Asturias of Applied Scientific Research and Technology (FICYT), Reina Sofía Institute for Nephrology Research, Fundación Renal Íñigo Álvarez de Toledo, RETIC RedInRen of the ISCIII - European Regional Development Fund (RD06/0016/1013, RD12/0021/1023 and RD16/0009), by the Asturian Society for Metabolic Research.

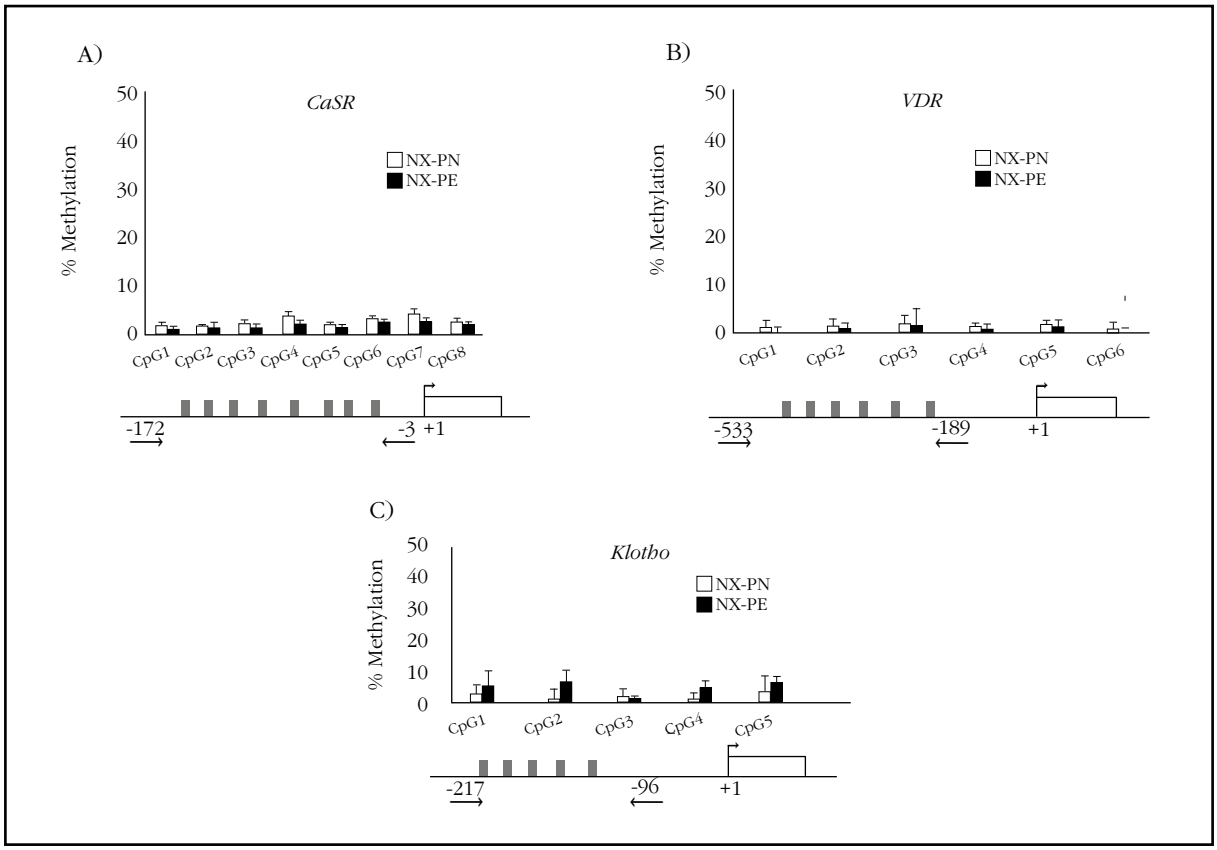
Conflict of interest: The authors affirm that they have no conflict of interests in this paper.

The handling of experimental animals has been carried out in accordance with the provisions of current legislation (European Union Directive 2010/63/EU and Royal Decree 53/2013 of 1 February).

Table 2. General biochemical markers and mineral metabolism

	Urea (mg/dL)	Creatinine (mg/dL)	Ca (mg/dL)	P (mg/dL)	PTH (pg/mL)	FGF23 (pg/mL)
NX-NP	104 ± 32	1.0 ± 0.3	11.7 ± 1.1	5.8 ± 1.2	44 ± 23	378 ± 103
NX-EP	201 ± 51	2.1 ± 0.4	10.5 ± 1.1	12.8 ± 1.9	1,762 ± 493	1,029 ± 101
P value	0.001	0.001	0.066	0.001	0.001	0.001

Figure 1. Degree of methylation of the CpGs sites preceding the initiation of promoter transcription of the A) CaSR (-172 to -3) genes; B) VDR (-533 to -189) and C) α-Klotho (-217 to -96) in parathyroid glands from nephrectomized (NX) rats fed a diet with normal phosphorus content PN (NX-PN) or high (NX-PE) for 20 weeks. At the bottom of each gene, the analyzed area of the promoter and the number of CpG sites present in the fragment are plotted

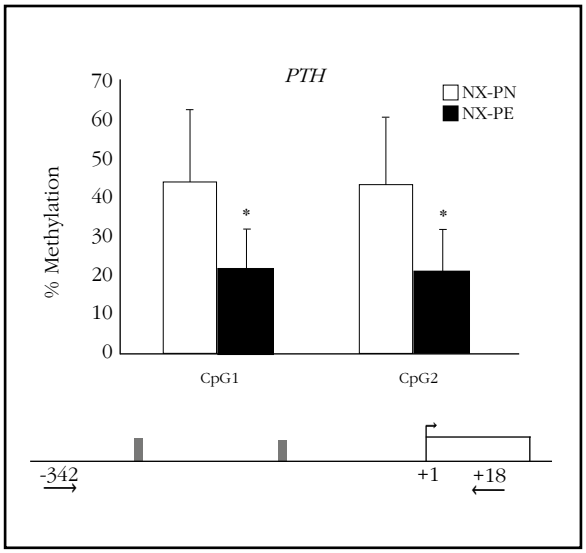


Bibliography

1. Eckardt KU, Coresh J, Levin A. Evolving important of kidney disease: from subspecialty to global health burden. Lancet. 2013;382:158-69.
2. Silver J, Levi R. Cellular and molecular mechanisms of secondary hyperparathyroidism. Clin Nephrol. 2005;63:119-26.
3. Rodriguez M, Cañadillas S, Lopez I, Aguilera-Tejero E, Almaden Y. Regulation of parathyroid function in chronic renal failure. J Bone Miner Metab. 2006;24:164-8.
4. Hu MC, Kuro-o M, Moe OW. The emerging role of Klotho in clinical nephrology. Nephrol Dial Transplant. 2012;27:2650-7.
5. Korkor AB. Reduced binding of [3H]1,25-dihydroxyvitamin D3 in the parathyroid glands of patients with renal failure. N Engl J Med. 1987;316:1573-7.
6. Ritter CS, Finch JL, Brown AJ. Parathyroid hyperplasia in uremic rats precedes down-regulation of the calcium receptor. Kidney Int. 2001;60:1737-44.
7. Carmona FJ, Esteller M. Epigenomics of human colon cancer. Mutat Res. 2010;693:53-60.
8. Kim MS, Lee J, SidranskyD. DNA methylation markers in colorectal cancer. Cancer Metastasis Rev. 2010; 29:181-206.
9. Ng JM, Yu J. Promoter hypermethylation of tumour suppressor genes as potential biomarkers in colorectal cancer. Int J Mol Sci. 2015;16:2472-96.
10. Kim Y, Kim DH. CpG island hypermethylation as a biomarker for the early detection of lung cancer. Methods Mol Biol. 2015;1238:141-71.
11. King GD, Rosene DL, Abraham CR. Promoter methylation and age-related downregulation of Klotho in rhesus monkey. AGE. 2012;34:1405-19.
12. Sun CY, Chang SC, Wu MS. Suppression of Klotho

expression by protein-bound uremic toxins is associated with increased DNA methyltransferase expression and DNA hypermethylation. Kidney Int. 2012;81:640-50.
13. Levine MA, Morrow PP, Kronenberg HM, Phillips III JA. Tissue and gene specific hypomethylation of the human parathyroid hormone gene: association with parathyroid hormone gene expression in parathyroid glands. Endocrinology. 1986;119:1618-24.
14. Naves Díaz M, Carrillo-López N, Rodríguez-Rodríguez A, Braga S, Fernández-Coto MT, López-Novoa JM, et al. Differential effects of 17ß- estradiol and raloxifene on bone and lipid metabolism in rats with chronic kidney disease and estrogen insufficiency. Menopause. 2010;17:766-71.
15. Varshney S, Bhadada SK, Sachdeva N, Arya AK, Saikia UN, Behera A, et al. Methylation status of the CpG islands in vitamin D and calcium-sensing receptor gene promoters does not explain the reduced gene expressions in parathyroid adenomas. J Clin Endocrinol Metab. 2013;98:E1631-5.
16. Hofman-Bang J, Gravesen E, Olgaard K, Lewin E. Epigenetic methylation of parathyroid CaR and VDR promoters in experimental secondary hyperparathyroidism. Int J Nephrol. 2012;2012:123576.
17. Uchiyama T, Tatsumi N, Kamejima S, Waku T, Ohkido I, Yokoyama K, et al. Hypermethylation of the CaSR and VDR genes in the parathyroid glands in chronic kidney disease rats with high-phosphate diet. Human Cell. 2016;29:155-61.
18. Azuma M, Koyama D, Kikuchi J, Yoshizawa H, Thasinas D, Shiizaki K, et al. Promoter methylation confers kidney-specific expression of the Klotho gene. FASEB J. 2012;26:4264-74.
19. Almaden Y, Hernandez A, Torregrosa V, Canalejo A, Sabate L, Fernandez Cruz L, et al. High phosphate level directly stimulates parathyroid hormone secretion and synthesis by human parathyroid tissue in vitro. J Am Soc Nephrol. 1998;9:1845-52.
20. Moallem E, Kilav R, Silver J, Naveh-Many T. RNA-protein binding and post-transcriptional regulation of parathyroid hormone gene expression by calcium and phosphate. J Biol Chem. 1998;273:5253-9.
21. Silver J, Kilav R, Naveh-Many T. Mechanisms of secondary hyperparathyroidism. Am J Physiol Renal Physiol. 2002;283:F367-76.
22. Takahashi F, Denda M, Finch JL, Brown AJ, Slatopolsky E. Hyperplasia of the parathyroid gland without secondary hyperparathyroidism. Kidney Int. 2002;61:1332-8.

Figure 2. Degree of methylation at the CpGs sites of the -342 to +18 region of the PTH promoter preceding the initiation of transcription in parathyroid glands from nephrectomized (NX) rats fed a diet with normal phosphorus content (NX PN) or high (NX-PE) for 20 weeks. (*P<0.05 with respect to NX-PN). At the bottom of the gene, the analyzed area of the promoter and the number of CpG sites present in this fragment



Montoya MJ[1], Vázquez MA[1], Miranda C[2], Miranda MJ[2], Pérez-Cano R[1,2], Giner M[1,3]

1 Departamento de Medicina - Facultad Medicina - Universidad de Sevilla - Sevilla (España)

2 Unidad de Osteoporosis - Servicio Medicina Interna - Hospital Universitario Virgen Macarena - Sevilla (España)

3 Departamento de Citología Normal y Patológica - Facultad de Medicina - Universidad de Sevilla - Sevilla (España)

Influence of vitamin D on biomechanical microstructure and properties of patients with hip fracture

DOI: http://dx.doi.org/10.4321/S1889-836X2017000400004

Correspondence: Mª José Montoya - Avda. Dr. Fedriani, s/n - 41009 Sevilla (Spain)
e-mail: pmontoya@us.es

Date of receipt: 31/03/2017
Date of acceptance: 21/05/2017

I work for the FEIOMM to attend the 35th Congress of the ASBMR (Baltimore, 2013).

Summary

Introduction: To assess serum levels of 25-hydroxyvitamin D-25 (OH) D-hormones with influence on bone metabolism (parathormone -PTH- and insulin-like growth factor (IGF)-I), bone remodeling markers (BRM) (carboxy-terminal telopeptide of collagen type I-β-CTX- and amino-peptide pro-peptide of procollagen type I -PINP), bone mineral density (BMD), microstructure and biomechanics of the femoral neck, in patients with osteoporotic hip fracture (OH) versus arthritic patients (OA).

Material and methods: A cross-sectional observational study of 29 OH and 14 OA, age ≥50 years. We quantified hormonal serum levels and BRM (immunoassay), hip BMD (DXA), microstructure (micro-CT) and biomechanics (uniaxial compression tests, IGFA system). Analysis (SPSS 20.0.)

Results: OH patients had lower levels of 25(OH)D ($p=0.02$) and hip BMD ($p<0.05$), and higher PTH ($p=0.029$) and β-CTX ($p=0.04$). Levels of 25(OH)D correlated positively with IGF-I ($p=0.04$) and negatively with β-CTX ($p=0.003$). The PTH values were correlated negatively with hip BMD ($p=0.0005$) and positively with trabecular thickness (TbTh) ($p=0.006$). Patients with 25(OH)D <20 ng/mL presented higher levels of β-CTX ($p=0.006$), lower IGF-I ($p=0.007$) and TbTh ($p=0.04$).

Conclusions: Vitamin D levels are low in the elderly population, especially in patients with osteoporotic hip fracture. These patients also presented raised levels of PTH and BRM and descended from BMD. Patients whose 25(OH)D levels are below 20 ng/mL present higher bone remodeling, with lower levels of IGF-I and alterations of the bone structure (TbTh) that may be linked to a greater risk of fractures.

Key words: *vitamin D, osteoporosis, hip fracture, bone mineral density, microstructure and biomechanics.*

Introduction

Hip fracture is one of the main and most dreaded complications of osteoporotic disease. Among the risk factors that favor this type of fracture include a greater tendency to fall and a decrease in bone strength. Bone mineral density (BMD), the rate of bone remodeling, geometry, microstructure and bone tissue mineralization are fundamental properties associated with bone resistance[1].

Vitamin D is known to be essential, among other factors, in the maintenance of musculoskeletal health. Proper levels of 25-hydroxivitamin D (25(OH)D) are needed to maintain the homeostasis of calcium metabolism and an insufficiency of these leads to a lower intestinal absorption of calcium, decreased levels of serum calcium, increased secretion of PTH, excessive rate of bone remodeling and, therefore, a lower amount and bone quality[2]. Furthermore, levels below 30 ng/mL have been found to be associated with significant defects of bone mineralization and increase in the osteoid substance[3]. All of these disorders produce a decrease in bone strength and, thus, a greater risk of fracture. As for the muscle, several studies have shown the positive relationship between 25(OH)D levels and muscle strength, especially in the lower extremities in the elderly[4,5]. Other research indicates muscular weakness and pain, as characteristic symptoms of Vitamin D-deficient syndromes[6], as well as increased muscle strength and balance and reduced risk of falls following the administration of adequate vitamin D supplements[7-10]. Two meta-analyzes evaluating controlled, double-blind and randomized studies conclude the beneficial effect of vitamin D supplementation, with a 19% reduction in falls, an 18% risk of hip fracture and 20% of the risk of any type of non-vertebral fracture[11,12]. These same studies pointed out that anti-fall and antifracture efficiency of 25(OH) D is achieved when their levels are above 24 and 30 ng/mL, respectively.

In the adult population, a high frequency of inadequate levels of vitamin D has been reported, below a variable threshold that ranges between 20-40 ng/mL of 25(OH)D, according to the different authors, in several studies carried out in multiple communities in Europe and the United States[13-15]. In Spain, this has also been observed in the elderly population with different characteristics in different regions, with values below 15 ng/mL being described in 68% of 77-year-olds living at home, compared to 100% of those institutionalized in Andalusia[16], a somewhat lower frequency in Cantabria[17], and even lower levels (4.6 ng/mL as mean values) in patients with hip fracture in Madrid[18].

These data reveal, on the one hand, the importance of vitamin D on muscle health and skeletal integrity and, on the other hand, the frequency of insufficient levels of the hormone among the adult population in general. However, the impact of serum vitamin D levels on the microstructural and biomechanical characteristics of bone tissue in patients with hip fracture has not been reported to date. Therefore, the main objective of our study is to ascertain if vitamin D levels influence the microarchitecture and biomechanical properties of the bone tissue. Secondly, we also analyze the association of these levels with biochemical markers of bone remodeling and with hormones that influence bone formation and resorption (parathormone -PTH-, and insulin-like growth factor -IGF-I-, respectively) parameters, all of which contribute to bone quality and, therefore, the risk of osteoporotic fracture.

Material and methods

1. Patients

A cross-sectional observational study of 43 patients aged 50-93 years who underwent hip arthroplasty. The first group consisted of 29 (6 male and 23 female) patients with nontraumatic hip fracture (OP), considered as idiopathic (involutive) osteoporosis. The second, as a comparative group, consisted of 14 patients (6 men and 8 women) with osteoarthritis (OA) and values of bone mass, at the hip level, T-score >(-2.5), with no personal history of fracture Osteoporosis or disease with influence on bone metabolism. Normal renal function was required.

All patients were recruited from the Traumatology and Orthopedics Service during the period from June 2014 to June 2015. Blood samples for biochemical determinations were collected in pairs with cases and controls in order to avoid seasonal bias in results. The protocol was reviewed and approved by the Center's Ethics Committee and all patients gave their informed consent.

The following data were collected: age, weight, body mass index (BMI), toxic habits (alcohol intake and smoking), semi-quantitative calcium intake (1 glass of milk=200 mg/day, 1 milk derivative=200 mg/day, 1 serving of cheese=200 mg/day), first-degree family history with osteoporotic fracture, treatment with vitamin D supplements and/or antiresorptive drugs. Bone mass was evaluated in the contralateral hip in the period between 15-30 days after surgery.

Bone samples were collected from the extracted femoral head for microstructural analysis and study of bone biomechanical properties after compression test.

2. Biochemical parameters

To carry out the biochemical determinations, blood samples were taken from the patients, fasting, in the first 48 hours after surgery. The following parameters, related to bone metabolism, were analyzed: calcium corrected for protein levels, phosphorus, reabsorption bone remodeling markers (carboxyl-terminal telopeptide of type I or β-CTX collagen) and formation (amino terminal propeptide of procollagen type I or PINP, parathormone (PTH), 25-hydroxyvitamin D (25 (OH) D) and insulin-like growth factor (IGF-I).

Serum levels of corrected calcium and phosphorus were assessed using the DAX-96 autoanalyzer. The levels of β-CTX and PINP were analy-

zed by immunoassay (electro-chemiluminescence), with the autoanalyzer COBAS e 601 (Roche, Spain), with coefficients of variation (CV) being interassayed <7.6% and <4.2%, respectively. Serum PTH was measured by immunoassay (electro-chemiluminescence), with autoantibody ADVIA Centaur (Siemens, Germany), with the CV interassay <5.8%. Serum 25(OH)D was analyzed by direct competitive immunoassay (electro-chemiluminescence), with the autoanalyzer LIAISON (DiaSorin, Italy), with the interassay CV <5.5%. Finally, serum IGF-I was quantified by immunoassay (electro-chemiluminescence), with the autoanalyzer IMMULITE (Siemens, Germany), with CV interassay being <3.9%.

3. Bone mass evaluation

Bone mineral density of hip and contralateral femoral neck was quantified using dual X-ray densitometry (DXA), with a Hologic-Densitometer (Hologic Inc.). The CV in vivo was 1% (total BMD).

4. Microstructural and biomechanical study of bone tissue

The analysis of the microstructure and the biomechanical properties has been made from the bone samples taken at the time of the surgical intervention for the prosthesis collation. From each femoral head, a trabecular bone cylinder from the primary compression region has been extracted with the longitudinal axis of the cylinder aligned with the main trabecular direction (MTD) (Figure 1).

Microstructural analysis of the biopsies has been carried out using computerized microtomography (micro-CT) using Bruker SkyScan 1172. The scanning of the sample was carried out at a resolution of 11 m, taking 2 images for each step of sample rotation (0.40°/pass, 180° total rotation). The images obtained have been reconstructed using the modified Feldkamp algorithm and later used for the quantitative and qualitative analysis of the trabecular bone microstructure.

The quantitative variables were: bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), degree of anisotropy (DA), which is a measurement of the symmetry of the object, or the presence/absence of structures aligned in a given direction, model structure index (MSI), indicating the relative prevalence of trabeculae in the form of plates or in the form of tube-cylinder and connectivity (trabecular pattern factor, Tb.Pf), which is an index of inverse connectivity, so that the higher the value the less connected the trabeculae are.

To assess biomechanical properties, uniaxial compression tests were carried out using the IGFA (image-guided failure analysis), applying a maximum force of 200 N, without reaching the elastic resistance limit of the sample. The variables that were quantified to describe the mechanical behavior of the bone tissue were the maximum stress (σ), or maximum internal resistance of the object to a force acting on it. The maximum deformation (ε), which represents the changes in the dimensions of the object subjected to the action of force, from which microfractures occur and Young's modulus, or elastic modulus, which represents the slope of the elastic region.

5. Statistical analysis

The IBM SPSS statistical package version 20.0 (USA) was used. For the statistical analysis of the results of quantitative variables, we carried out comparisons of Student's t-means for independent samples. We did univariate linear analysis (ANCOVA) to take into account possible confounding factors such as age and BMI. Pearson's correlation test was used to assess the association between variables. To study the qualitative variables, we have analyzed contingency tables, χ^2. In all cases, a level of $p < 0.05$ was required to consider significant differences.

Results

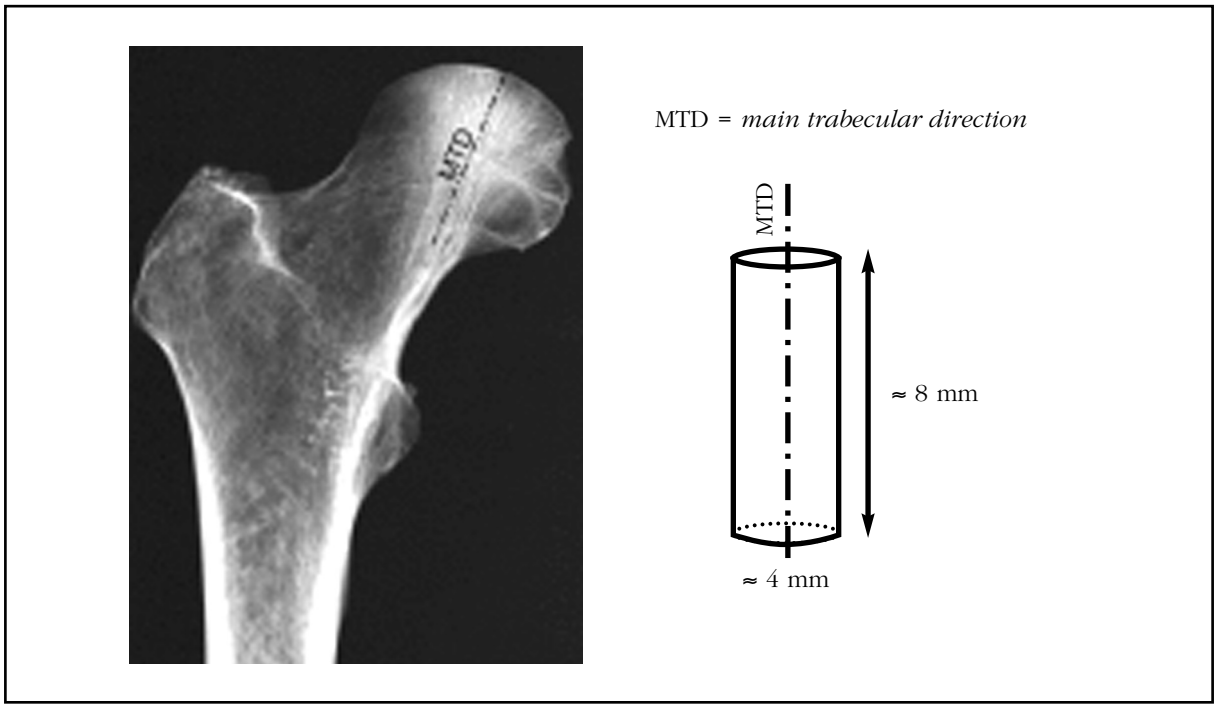
The characteristics of the patients studied in each of the groups are shown in table 1. As expected, patients with hip fracture had a significantly higher age, 81±8 vs 68±9 years, p=0.001, a lower BMI 28.2±5.8 vs 31.9±4.4, p=0.043 and lower hip mass values (p<0.05 for all locations). Since the group of patients with fracture and osteoarthrosis were different in terms of age and BMI, the parameters analyzed were adjusted for these variables. In terms of habits, family history of first-degree osteoporosis, treatment with antiresorptive drugs and supplementation with vitamin D and calcium were similar in both groups.

1. Serum values of parameters related to bone metabolism

The biochemical parameters of the patients studied are shown in table 2. Serum levels of 25(OH)D were significantly lower in patients with fracture than in the group of patients with osteoarthritis (10.9±6.9 vs 18.1±10.7 ng/mL, p=0.02). The values of PTH (60±41.1 vs 38.2±17.3 pg/mL, p=0.029) and the bone resorption marker β-CTX (0.61±0.26 vs 0.36±0.19 ng/mL, p=0.04) were significantly higher in the first group. Levels of corrected serum calcium, phosphorus, bone formation marker PINP and IGF-I were comparable in both groups. After adjustment for age and BMI, we verified that the differences that we found for 25(OH)D and β-CTX remained significant. The adjusted values for 25(OH)D were 10.7±(95% CI 6.6-14.7) ng/mL for the hip fracture group and 19.6±95% CI, 7-25.5) ng/mL, for that of arthrosic patients, (p=0.027). The levels of β-CTX adjusted for the same variables were 0.63±(95% CI 0.51-0.7) ng/mL, in the group of patients with hip fracture and 0.30±95% 0.1-0.5) ng/ml in the arthrosis group (p=0.012).

Levels of 25(OH)D showed a significant and positive correlation with serum IGF-I (r=0.338, p = 0.044) and negative with β-CTX levels (r=-0.483, p=0.003). PTH levels were significantly negatively correlated with BMD-hip (r=-0.617, p=0.005) and positively with trabeculae separation (r=0.530, p=0.006).

Figure 1. Localization of the trabecular bone cylinder extraction for the analysis of the microstructure and biomechanical properties



2. Bone tissue microstructure and biomechanics
Microstructural indices indicate lower bone quality in the group of patients with hip fracture than the arthrosic group. In figure 2, we show the result of scanned images of two patients, each belonging to a study group.

Although no parameter presented a statistically significant difference, we verified that the values of percentage of bone volume, as well as the thickness and number of trabeculae were lower in patients with fracture, whereas the separation between them was greater (Table 3).

The trabecular pattern index (inverse index of connectivity) presented higher values in the group of fractured patients. The structure index of the model, which implies a relative prevalence of trabeculae in the form of tube-cylinder compared to those of plate form, was higher in the group of patients with hip fracture, indicating a greater number of trabeculae in the form of a tube-cylinder, less resistant in these patients.

Biomechanics studies showed that Young's modulus values, maximal strain and maximum deformation, after applying compression tests, were lower in patients with hip fracture than in patients with osteoarthritis, although these differences were not significant (Table 3).

3. BMD and bone structure results in patients with insufficient levels of 25(OH)D (<20 ng/mL)
Serum levels of 25(OH)D in the studied population have shown very low values, in the range of 4.0-41.6 ng/mL when dividing patients into two groups, considering that these levels were less than or equal to 20 ng/mL or higher, we found the following results: the two populations were similar in age, weight, BMI, bone mass values and biomechanical characteristics of bone tissue. However, those with levels below 20 ng/mL had higher serum β-CTX values (0.58±0.25 vs 0.30±0.15 ng/mL, p=0.006), lower IGF-I (49.8±27.0 vs 83.5±35.6 ng/mL, p=0.007) and the bone structure showed a smaller trabecular width (0.34±0.17 vs 0.50±0.1 mm, p=0.04) (Figure 3).

Discussion

This study allowed us to compare serum levels of vitamin D in patients with and without osteoporotic hip fracture and the association of these levels with markers of bone remodeling, hormones regulating bone metabolism, BMD, microstructural indexes and biomechanical properties of the femoral neck in these patients.

We found that patients with osteoporotic hip fracture had significantly lower serum 25(OH)D levels than non-fractured patients. In addition, we noted a high prevalence in elderly people with deficient levels (<20 ng / mL), especially in the OP group, in line with what was observed by other authors[13-18], despite having approximately 3,000 hours of sunshine per year in our environment. As is known, insufficient levels of vitamin D are associated with an increased risk of falls and osteoporotic fractures[11,12]. In our study, we also verified that patients with osteoporotic hip fracture also present higher serum levels of β-CTX and PTH, together with lower BMD values in all hip-measured locations, compared to patients without fracture. In addition, we demonstrated a significant negative correlation between 25(OH)D and β-CTX levels, as well as between PTH and BMD levels.

Table 1. Characteristics of the study population. Values expressed as mean ± SD

	OA (n=14)	OP (n=29)	p
Age (years)	68±9	81±8	p=0.0001
Weight (kg)	77.8±17.9	68.7±13.5	n.s.
BMI (kg/m²)	31.92±4.41	28.22±5.8	p=0.043
BMD neck (g/cm²)	0.733±0.15	0.577±0.09	p=0.008
BMD hip (g/cm²)	0.905±0.14	0.774±0.12	p=0.029
T-score neck	-1.3±1.1	-2.5±0.8	p=0.008
T-score hip	0.7±0.8	-1.5±0.9	p=0.034
Smoking	14.3%	11.5%	n.s.
Family history in 1st degree of osteoporosis	23.1%	30.8%	n.s.
Vitamin D treatment	23.1%	10.5%	n.s.
Intake of calcium (mg/day)	770.4±444.8	725.1±252.6	n.s.

n.s.: not significant.

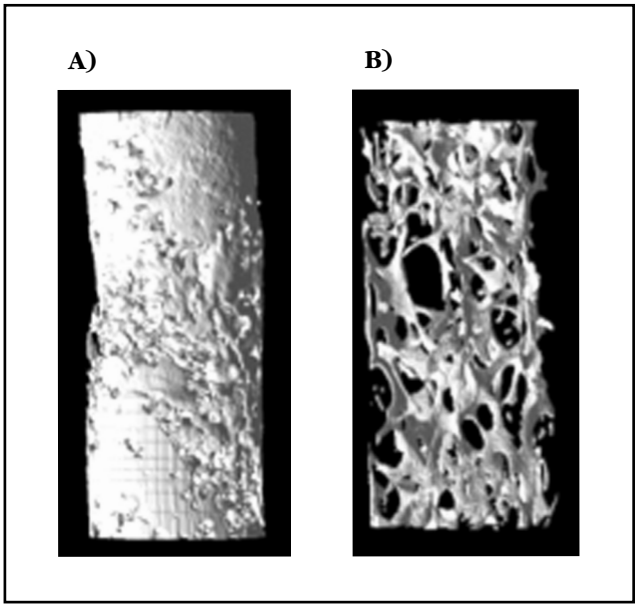
Table 2. Serum values of biochemical parameters of the study population. Values expressed as mean ± SD

	OA (n=14)	OP (n=29)	p
25(OH)D (ng/mL)	18.1±10.7	10.9±6.9	p=0.02
PTH (pg/mL)	38.2±17.3	60±41.1	p=0.029
PINP (ng/mL)	55.8±37.6	57.6±38.0	n.s.
β-CTX (ng/mL)	0.36±0.19	0.61±0.26	p=0.04
Phosphorus (mg/dL)	3.1±0.5	3.1±0.8	n.s.
Corrected calcium (mg/dL)	9.5±0.6	9.4±0.4	n.s.
IGF-I (ng/mL)	66.3±36.6	51.7±29.0	n.s.

n.s.: not significant.

Taken together, these data once again explain that low levels of 25(OH)D are associated with high levels of PTH, that inducing a greater bone turnover would explain the higher values of bone remodeling markers. The imbalance in the levels of these parameters causes trabecular deterioration that could lead to increased risk of fracture. A high rate of bone remodeling increases the number of resorption cavities. These cavities act as "areas of stress accumulation", foci of weakness that may increase the risk of microfractures and macrofractures[19]. Excessive resorption may also lead to perforation of the trabeculae and permanent loss of connectivity. All these alterations described at the microstructural level of the trabecular bone make it less resistant to the load and, therefore, with an increased risk of fracture[20]. In our study, all individuals with non-traumatic hip fracture and with osteoarthritis, except one in the latter group, had levels of 25(OH)D <40 ng/mL. Some authors report that men with radiologi-

Figure 2. Reconstruction of scanned images of patient biopsies with osteoarthritis (A) and hip fracture (B)



cal osteoarthritis of the hip have lower levels of 25(OH)D and a prevalence of deficiency higher than the control population[21]. It is generally known that up to this level of 40 ng/mL an inverse relationship between vitamin D and PTH[16] is described. Although a low calcium intake may also be responsible for increasing PTH levels, in our case we found that calcium intake was low but similar in both study groups (<800 mg/day). Although this is a factor to be taken into account, we know that insufficient levels of vitamin D lead to elevated levels of PTH, despite adequate calcium intake, and conversely, sufficient levels of vitamin D maintain normal levels of PTH despite calcium intake <800 mg/day[22].

Although patients with hip fracture presented lower BMD values than patients with osteoarthritis at all hip-measured locations, we did not find an association between these values and serum levels of 25(OH)D, as reported by other authors[23]. However, we would like to point out that when all patients studied according to their 25(OH)D levels were <20 ng/mL or higher, the group of patients with lower levels also had lower levels of hip BMD, of 0.06 gHA/cm^2. These data may not be important at the individual level, but in terms of population level, taking into account each decrease of 1 standard deviation (SD), the risk of hip fracture increases by an average of 2.6 times[24,25]. This would imply that, with the lowest bone mass found, the risk of fracture may be increased up to 1.5 times.

Many experts, based on broad population studies, have jointly pointed out that minimum levels of 25(OH)D of at least 20-30 are desirable to maintain overall health integrity, and bone in particular, Ng/mL (50-75 nM)[26-29]. The negative effect of insufficient levels of vitamin D on bone has been assessed by surrogate factors such as PTH, BMD, and markers of bone remodeling. It has also been directly analyzed through its association with bone properties involved in bone strength and, therefore, the risk of fractures. In this sense, it has been verified by histo-morphometric studies of iliac crest that vitamin D insufficiency is associated with mineralization defects, showing higher levels of surface and volume of osteoid, and concluding that hormone levels higher than 30 Ng/mL to prevent pathological accumulation of osteoid[3]. One of the fundamental aspects of our study is the assessment of microarchitecture and biomechanical properties of the femoral neck of patients with and without osteoporotic hip fracture. Although the differences in the different parameters were not significant in any of the cases, as has been pointed out by other authors[30,31], we found that patients with hip fracture presented a percentage of bone volume (BV/TV), a number of trabeculae (Tb.N) and a width of these (Tb.th) 16%, 15% and 13% lower, respectively, than those of patients with osteoarthritis. In addition, the trabeculae were less connected and predominant than those presented as cylinder-tube in the fractured group, which is related to a lower bone resistance[32]. Along with this, biomechanical parameters also showed results between 10-14% lower in patients with hip fracture. We would like to highlight, as the most important point of this study, that it analyzes for the first time the serum levels of vitamin D, directly related to the microstructural properties of bone tissue in people with hip fracture. When comparing hormone levels among patients, we found that levels below 20 ng/mL were associated with a significant reduction in trabecular width, in addition to higher levels of β-CTX, indicating a greater activity of bone resorption, together with

Table 3. Microstructure and biomechanical bone properties of the study group. Values expressed as mean ± SD

	OA (n=14)	OP (n=29)
BV/TV (%)	36.2±12.1	30.5±12.9
Tb.th (mm)	0.39±0.2	0.34±0.2
Tb.Sp (mm)	1.02±0.6	1.04±0.5
Tb.N (mm-1)	0.98±0.9	0.83±0.7
Tb.Pf (mm-1)	-0.08±4.2	2.81±5.0
SMI	0.44±1.3	1.07±0.8
AD	2.96±0.67	3.43±0.98
Módulo de Young (Mpa)	460±276	397±173
σ (Mpa)	9.1±4.9	8.2±3.6
ε	0.045±0.04	0.039±0.02

BV/TV: bone volume fraction; Tb.th: trabecular thickness; Tb.Sp: trabecular separation; Tb.N: number of trabeculae; Tb.Pf: trabecular connectivity; SMI: structural model index; AD: degree of anisotropy; σ: maximum voltage; ε: maximum deformation.

lower levels of IGF-I. Vitamin D status reportedly contributes to the determination of serum IGF-I levels[33]. In turn, IGF-I has been shown to stimulate renal 1 -hydroxylase activity, contributing to phospho-calcium metabolism, in addition to benefitting bone mass[34], associated with its reduction to a decrease in bone formation that contributes to bone loss in senile osteoporosis[35].

Our study has several limitations, especially in terms of sample size, which is relatively small. Furthermore, the dispersion of results of structural and mechanical parameters could be remedied, at least in part, with larger studies.

Overall, given the results obtained, we may conclude that the elderly population has a deficient vitamin D status, that these levels are even lower among patients with osteoporotic hip fracture and that serum concentrations below 20 ng/mL of the hormone can, directly or indirectly through PTH and IGF-I, condition an alteration in bone remodeling (β-CTX elevation) and BMD, with a consequent repercussion at the microstructural level (Tb.Th, among others) that lead to it being a low resistance bone where fractures easily occur.

Conclusions

These results indicate that patients with hip fracture have lower levels of 25(OH)D than patients with osteoarthritis, and that these induce raised PTH and increased bone resorption (with consequent increase in β-CTX levels) leading to decreased bone mass, decreased quality and increased risk of fracture. These alterations are more pronounced in patients with serum levels of 25(OH)D <20 ng/mL, in which there is also a decrease in IGF-I and trabecular width at the structural level of the femoral neck trabecular bone.

Acknowledgements: Our sincere thanks to all the participants in the study and to the staff of the Unit of Management of Orthopedics and Traumatology of the Macarena University Hospital Orthopedics and Traumatology Department (Spain). We are also grateful for the funding of Project PI13/00702, the Carlos III Health Institute, the 2013-2016 National Plan and the ERDF and ISCIII 2013-RED 12-0043 (RETICEF), which have made it possible for this study to be carried out.

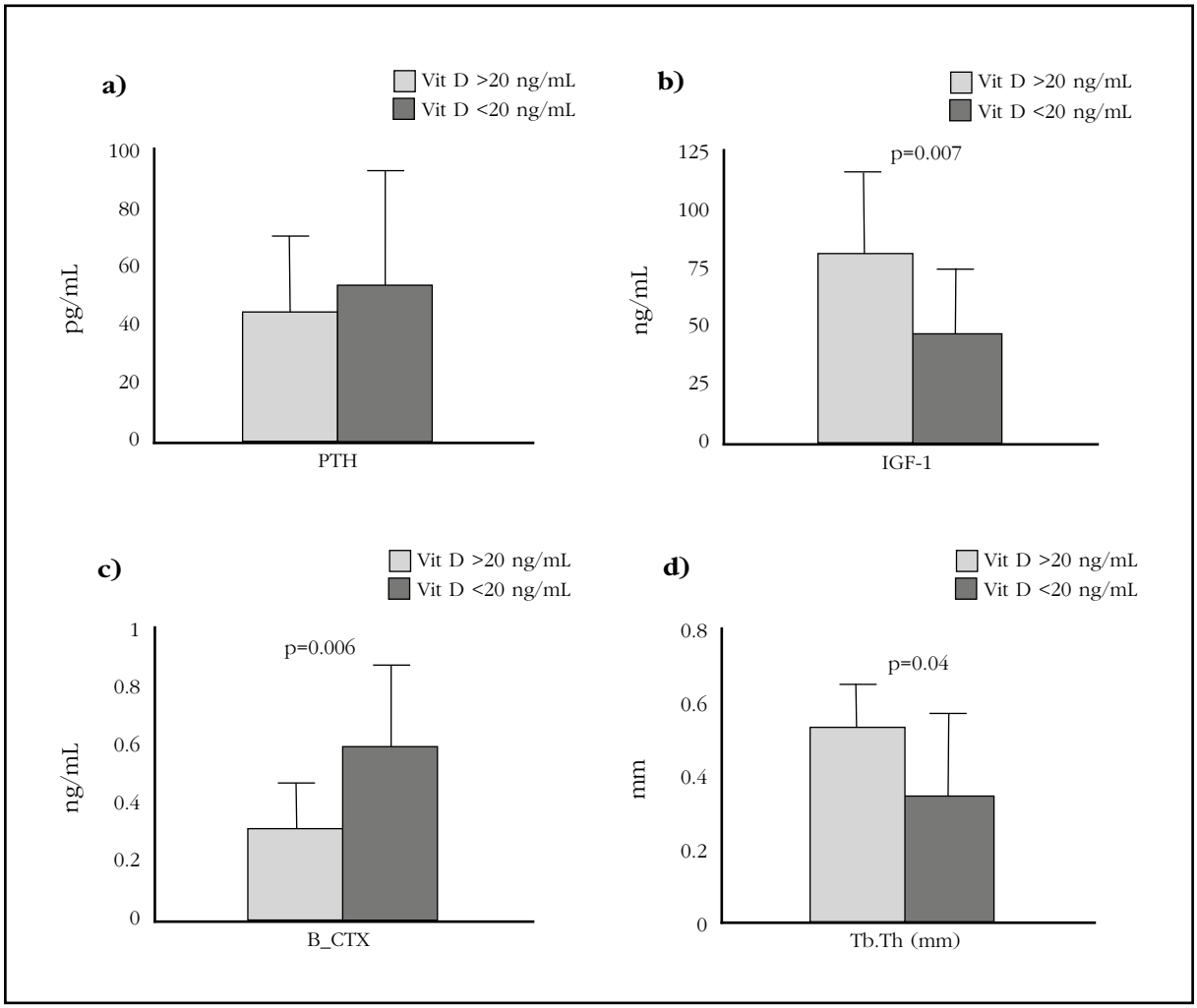
Conflicts of interest: None of the authors have a conflict of interest.

Ethics: The study was approved by the Institutional Ethics Committee (Macarena University Hospital, Seville, Spain) and informed consent was obtained from each participant.

Bibliography

1. Veldurthy V, Wei R, Oz L, Dhawan P, Jeon YH, Christakos S. Vitamin D, calcium homeostasis and aging. Bone Res. [Internet]. 2016;4:16041.
2. Sosa Henríquez M, Gómez de Tejada Romero MJ, Recker RR, Cannata Andía JB, Del Pino Montes J, Díaz Curiel M, et al. Papel del calcio y la vitamina D en el tratamiento de la osteoporosis. Rev Osteoporos Metab Miner. 2010;2(1):61-72.
3. Priemel M, von Domarus C, Klatte TO, Kessler S, Schlie J, Meier S, et al. Bone mineralization defects and vitamin D deficiency: histomorphometric analysis of iliac crest bone biopsies and circulating 25-hydroxyvitamin D in 675 patients. J Bone Miner Res. 2010;25(2):305-12.
4. Wicherts IS, van Schoor NM, Boeke a JP, Visser M, Deeg DJH, Smit J, et al. Vitamin D status predicts physical performance and its decline in older persons. J Clin Endocrinol Metab. 2007;92(6):2058-65.
5. Bischoff-Ferrari HA, Dietrich T, Orav EJ, Hu FB, Zhang Y, Karlson EW, et al. Higher 25-hydroxyvitamin D concentrations are associated with better lower-extremity function in both active and inactive persons aged 60 y. Am J Clin Nutr. 2004;80(3):752-8.
6. Schott GD, Wills MR. Muscle weakness in osteomalacia. Lancet. 1976;307(7960):626-9.
7. Annweiler C, Beauchet O. Questioning vitamin D status of elderly fallers and nonfallers: a meta-analysis to address a 'forgotten step'. J Intern Med. 2015;277(1):16-44.
8. Pfeifer M, Begerow B, Minne HW, Suppan K, Fahrleitner-Pammer A, Dobnig H. Effects of a long-term vitamin D and calcium supplementation on falls and parameters of muscle function in community-dwelling older individuals. Osteoporos Int. 2009;20(2):315-22.
9. Pfeifer M, Begerow B, Minne HW, Abrams C, Nachtigall D HC. Effects of a short-term vitamin D and calcium supplementation on body sway and secondary hyperparathyroidism in elderly women. J Bone Min Res. 2000;15(6):1113-8.
10. Broe KE, Chen TC, Weinberg J, Bischoff-Ferrari HA, Holick MF KD. A higher dose of vitamin d reduces the risk of falls in nursing home residents: a randomized, multiple-dose study. J Am Geriatr Soc. 2007;55(2):234-9.
11. Bischoff-Ferrari HA, Dawson-Hughes B, Staehelin HB, Orav JE, Stuck AE, Theiler R, et al. Fall prevention with supplemental and active forms of vitamin D: a meta-analysis of randomised controlled trials. BMJ. 2009;339:b3692.
12. Bischoff-Ferrari HA, Willett WC, Wong JB, Stuck AE, Staehelin HB, Orav EJ, et al. Prevention of nonvertebral fractures with oral vitamin D and dose dependency: a meta-analysis of randomized controlled trials. Arch Intern Med. 2009;169(6):551-61.
13. Kuchuk NO, van Schoor NM, Pluijm SM, Chines A, Lips P. Vitamin D status, parathyroid function, bone turnover, and BMD in postmenopausal women with osteoporosis: global perspective. J Bone Miner Res. 2009;24(4):693-701.
14. Bandeira F, Griz L, Dreyer P, Eufrazino C, Bandeira C, Freese E. Vitamin D deficiency: A global perspective. Arq Bras Endocrinol Metab. 2006;50(4):640-6.
15. Holick MF. The vitamin D epidemic and its health consequences. J Nutr. 2005;135(11):2739S-48S.
16. Quesada JM, Jans I, Benito P, Jimenez JA, Bouillon R. Vitamin D status of elderly people in spain. Age Ageing. 1989;18(6):392-7.
17. Castillo Suárez M, Sosa Henríquez M. Modificación de las hormonas calciotropas y los marcadores bioquímicos de remodelamiento óseo, en función de la edad y el sexo, en una población anciana institucionalizada. Rev Esp Geriatr Gerontol. 1998;33:349-56.
18. Alarcón T, González-Montalvo JI, Hoyos R, Diez-Sebastián J, Otero A, Mauleon JL. Parathyroid hormone response to two levels of Vitamin D deficiency is associated with high risk of medical problems during hospitalization in patients with hip fracture. J Endocrinol Invest. 2015;38(10):1129-35.
19. Geissler JR, Bajaj D, Fritton JC. American Society of Biomechanics Journal of Biomechanics Award 2013: cortical bone tissue mechanical quality and biological mechanisms possibly underlying atypical fractures. J Biomech. 2015;48(6):883-94.

Figure 3. Serum levels of PTH (a), IGF-I (b), β-CTX (c) and Tb.th -trabecular enlargement- (d) of patients with serum levels of 25(OH)D> 20 ng/ML (n=10) or ≤20 ng/mL (n=33)



20. van der Linden JC, Weinans H. Effects of microarchitecture on bone strength. Curr Osteoporos Rep. 2007;5(2):56-61.
21. Chaganti RK, Parimi N, Cawthon P, Dam TL, Nevitt MC, Lane NE. Association of 25-hydroxyvitamin D with prevalent osteoarthritis of the hip in elderly men: The osteoporotic fractures in men study. Arthritis Rheum. 2010;62(2):511-4.
22. Steingrimsdottir L, Gunnarsson O, Indridason OS, Franzson L, Sigurdsson G. Relationship between serum parathyroid hormone levels, vitamin D sufficiency, and calcium intake. JAMA. 2005;294(18):2336-41.
23. Bischoff-Ferrari HA, Dietrich T, Orav EJ, Dawson-Hughes B. Positive association between 25-hydroxy vitamin D levels and bone mineral density: a population-based study of younger and older adults. Am J Med. 2004;116(9):634-9.
24. Cummings SR, Browner W, Cummings SR, Black DM, Nevitt MC, Browner W, et al. Bone density at various sites for prediction of hip fractures. Lancet. 1993;341(8837):72-5.
25. Ensrud KE, Taylor BC, Paudel ML, Cauley JA, Cawthon PM, Cummings SR, et al. Serum 25-hydroxyvitamin D levels and rate of hip bone loss in older men. J Clin Endocrinol Metab. 2009;94(8):2773-80.
26. Dawson-Hughes B, Mithal A, Bonjour JP, Boonen S, Burckhardt P, Fuleihan GEH, et al. IOF position statement: Vitamin D recommendations for older adults. Osteoporos Int. 2010;21(7):1151-4.
27. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011;96(7):1911-30.
28. Gómez de Tejada Romero MJ, Sosa Henríquez M, Del Pino Montes J, Jódar Gimeno E, Quesada Gómez JM, Cancelo Hidalgo MJ, et al. Documento de posición sobre las necesidades y niveles óptimos de vitamina D. Rev Osteoporos Metab Miner. 2011;3(1):53-64.
29. Atkinson SA. The new dietary reference intakes from the Institute of Medicine for calcium and vitamin D. Perspect Infirm. 2011;8(5):5.
30. Blain H, Chavassieux P, Portero-Muzy N, Bonnel F, Canovas F, Chammas M, et al. Cortical and trabecular bone distribution in the femoral neck in osteoporosis and osteoarthritis. Bone. 2008;43(5):862-8.
31. Zhang ZM, Li ZC, Jiang LS, Jiang SD DL. Micro-CT and mechanical evaluation of subchondral trabecular bone structure between postmenopausal women with osteoarthritis and osteoporosis. Osteoporos Int. 2010;21(8):1383-90.
32. Cohen A, Liu XS, Stein EM, McMahon DJ, Rogers HF, LeMaster J, et al. Bone microarchitecture and stiffness in premenopausal women with idiopathic osteoporosis. J Clin Endocrinol Metab. 2009;94(11):4351-60.
33. Bogazzi F, Rossi G, Lombardi M, Tomisti L, Sardella C, Manetti L, et al. Vitamin D status may contribute to serum insulin-like growth factor I concentrations in healthy subjects. J Endocrinol Invest. 2010;34(8):200-3.
34. Bex M, Bouillon R. Growth hormone and bone health. Horm Res. 2003;60 Suppl 3:80-6.
35. Riggs BL. Endocrine causes of age-related bone loss and osteoporosis. Novartis Found Symp. 2002;242:244-7.

Carrillo López N[1], Tuñón LePoultel D[1,2], Quirós Caso C[1,3], Rodríguez I[1], Cannata Andía JB[1], Naves Díaz M[1]

1 Servicio de Metabolismo Óseo y Mineral - Instituto Reina Sofía de Investigación Nefrológica - Red de Investigación Renal del Instituto de Salud Carlos III (REDinREN del ISCIII) - Universidad de Oviedo - Oviedo (España)

2 Laboratorio de Bioquímica - Hospital 12 de Octubre - Madrid (España)

3 Laboratorio de Medicina - Hospital Universitario Central de Asturias - Oviedo (España)

Effect of supra-physiological calcitriol doses on protein expression of vascular smooth muscle cells

DOI: http://dx.doi.org/10.4321/S1889-836X2017000400005

Correspondence: Jorge Cannata Andía - Servicio de Metabolismo Óseo y Mineral - Hospital Universitario Central de Asturias - Avenida de Roma, s/n - 33011 Oviedo (Spain)
e-mail: cannata@hca.es

Date of receipt: 31/03/2017
Date of acceptance: 16/05/2017

Grant-awarded work funded by the FEIOMM in Basic Research 2010.

Summary

Introduction: Calcitriol, essential for maintaining calcium and phosphorus homeostasis in the body, may damage the vascular system in high doses, increasing the risk of calcification.

Objective: To assess the differential expression of proteins in vascular smooth muscle cells subjected to a supra-physiological dose of calcitriol.

Material and methods: Rat vascular smooth muscle cells (VSMC-R) were cultured in the presence of 10^{-7} M calcitriol for 10 days. The change of muscle to bone phenotype was assessed by alkaline phosphatase activity, immunocytochemistry, quantitative polymerase chain reaction in time (QPCR) and Western blot analysis. By means of two-dimensional electrophoresis and mass spectrometry was evaluated for the differential protein pattern in presence and absence of 10^{-7} M calcitriol.

Results: Exposure to a high dose of calcitriol decreased elastin gene expression and the protein and gene expression of α-actin protein, increased gene expression of osteocalcin and Runx2 and expression of osteoprotegerin protein. At the proteomic level, 10 differentially expressed proteins were identified, highlighting the increase in mitochondrial superoxide dismutase, cytoskeleton proteins, vesicle formation and inflammasome. On the contrary, there were 4 proteins that diminished expression, highlighting some of muscular type.

Conclusions: In a model of vascular smooth muscle cells submitted to a supra-physiological dose of calcitriol an increased expression of cytoskeleton proteins was observed. These proteins form matrix vesicles and participate in clearance of free radicals and in the inflammatory response. The loss of muscle phenotype was represented by decreased expression of typically muscle proteins.

Key words: *vascular calcification, calcitriol, proteomics.*

Introduction

Vascular calcification (VC) is a prevalent alteration in aging, which has been linked to an increase in vessel stiffness and an increased risk of cardiovascular death[1]. In the general population, progression and VC rate have been associated with an increased risk of fractures and osteoporosis[2], preceding vascular alterations to bone alterations observed later[3]. Different epidemiological studies have shown the relationship between alterations in bone metabolism, VC and increased mortality[4].

The mechanism by which VC occurs is complex. Initially, it was thought to be a passive process consisting of a simple precipitation of calcium and phosphorus in an appropriate microenvironment[5]. However, it is now known that, in addition to this passive process, there are active mechanisms that run along with the former. During these active processes, vascular smooth muscle cells (VSMC), due to certain calcification promoters, suffer a greater degree of apoptosis, form vesicles and finally change their phenotype of smooth muscle cells to osteoblast-like cells, inducing matrix formation and also attracting local factors that determine the mineralization process[1].

Vitamin D, which exerts its main physiological effect on calcium and phosphorus homeostasis, has traditionally been used to treat and prevent rickets and osteomalacia[6]. It is hydroxylated in the kidney by the action of 25-hydroxyvitamin D-1α hydroxylase giving rise to calcitriol, which is the most active physiological metabolite of this hormone[7]. For years, high-dose calcitriol has been known to induce increased calcification in VSMC[8], alkaline phosphatase activity and reduced regulation of parathyroid hormone-related peptide (PTHrP)[9]. Further studies have confirmed the calcification promoting effect of calcitriol by increasing calcium deposition in the aorta of the rat model with normal renal function[10] and by increasing Messenger RNA levels (mRNA) of bone proteins: Runt-related transcription factor 2 (Runx2), osteocalcin, osteoprotegerin (OPG), activator receptor for nuclear factor κβ (RANKL) and bone morphogenetic protein 4 (BMP4) in the aorta of nephrectomized rats[11,12].

Therefore, this study aimed to evaluate the differential expression of proteins produced with the calcifying effect of supra-physiological doses of calcitriol in an *in vitro* model of VSMC.

Material and methods

Induction of calcitriol vascular calcification

The rat aorta vascular smooth muscle cell line, SMAC-R (primary culture DPK-SMAC-R; Pharmakine), was cultured at 37°C in a humid atmosphere with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, Lonza) with 10% fetal bovine serum (FBS, HyClone® Thermo Scientific), 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine (Biochrom AG).

Upon reaching the necessary confluence (approximately 60%), the cells were cultured with DMEM F-12 culture medium supplemented with 0.1% bovine serum albumin (BSA) in the absence or presence of calcitriol (10^{-7} M, Sigma- Aldrich) for ten days, changing the culture with fresh medium every 48 hours. All experiments were carried out in triplicate and each condition in each experiment was done in triplicate.

Determination of alkaline phosphatase activity

VSMCs cultured in 24-well plates with or without 10^{-7} M calcitriol for 10 days were collected, and alkaline phosphatase activity was quantified by measuring the amount of hydrolyzed para-nitrophenol phosphate following the instructions of the kit used for its determination (BioAssay Systems).

Immunocytochemistry

To evaluate the vascular phenotype loss, the immunocytochemistry of α-actin was carried out. In order to do so, the VSMCs were grown on a specific plastic support (Thermanox) and then exposed to 10^{-7} M calcitriol for 10 days. The mouse monoclonal antibody against α-actin (CP-47, Calbiochem) and the Dako Real™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako) were used for this purpose.

Analysis of gene expression

For the analysis of gene expression by quantitative real-time PCR (qPCR) RNA was previously extracted from the cells using Tri Reagent (Ambion) following a standard protocol. From 2 µg of total RNA, the cDNA was obtained using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the manufacturer's instructions. To quantify the relative genetic expression, a thermocycler model Stratagene Mx3005P QPCR System (Agilent Technologies) and Taqman® reagents (Taqman® Universal PCR Master Mix, Applied Biosystems) were used. We used the Applied Biosystems assays corresponding to the following genes: α-actin (Rn01759928_g1), elastin (Rn01499782_m1), osteocalcin (Rn01455285_g1) and Runx2 (Rn01512296_m1). The results were normalized against 18S endogenous control (Eukaryotic 18S rRNA endogenous control reagent, Applied Biosystems). The interpretation of the data was performed using the threshold cycle comparison method (ΔΔCt)[13].

Protein study

Two-dimensional protein electrotrophrosis:

For the study of differential protein expression by two-dimensional electrophoresis, VSMC was cultured for 10 days in the absence or presence of 10^{-7} M calcitriol. Total protein extraction was performed by homogenizing the cells in a lysis buffer composed of 7 M urea, 2 M thiourea and 2% CHAPS. Proteins were purified and desalted using the Ready Prep Clean-up kit (Bio-Rad) and quantified by the Bradford assay[14].

The isoelectric focusing (separation of the proteins according to their isoelectric point) or first dimension was carried out in triplicate with 150 µg of proteins of each condition in strips of 24 cm

dehydrated polyacrylamide with a non-linear pH range 3-10 (IPG-Strips, GE Healthcare). At 24 hours prior to isoelectric focusing, the strips were rehydrated with DeStreak Rehydration Solution and 0.5% ampholytes (both from GE Healthcare). The isoelectric focusing was performed on Ettan IPGphor3 (GE Healthcare), and the strips were then washed with equilibration buffer (6 M urea, 75 mM Tris-HCL, 30% glycerol and 2% SDS) with two 15-minute washes, First with dithiothreitol (DTT) 1% and the second with 2.5% iodoacetamide, for reduction-alkylation of proteins.

The second dimension was performed by SDS-PAGE on 12% polyacrylamide gels in a multiple cuvette (GE Healthcare). After the electrophoresis, the gels were stained with 0.1% silver nitrate and digitized with a calibrated densitometer GS-800, analyzing the different intensity of the spots using the PDQuest software, both of Bio-Rad.

Analysis of differential protein expression by mass spectrometry:
Differentially expressed spots in the two culture conditions (absence or presence of calcitriol) were cut from the gel, sliced and washed out in incubation for 10 minutes with 30 mM potassium ferrocyanide and 100 mM sodium thiosulfate, and dehydrated in acetonitrile. Finally, they were digested with 13 ng/µL trypsin in 10 mM ammonium bicarbonate/10% acetonitrile for 24 hours at 37°C. Peptides obtained from trypsin digestion were identified by liquid chromatography (nanoHPLC, Applied Biosystems) and ion trap mass spectrometry (Q-TRAP, Applied Biosystems) (LC-MS/MS). Mass spectra of differentially expressed proteins were identified using the UniProtKB/Swissprot database (www.uniprot.org) and the Mascot search engine.

Protein analysis by Western blot:
Western blot analysis was used to confirm the results obtained in two-dimensional electrophoresis. To this end, the total proteins were extracted from the cultures in RIPA lysis buffer and quantified by the Bradford standard method (Bio-Rad).

The different protein extracts (30 µg) were electrophoresed on polyacrylamide gels under denaturing conditions (SDS-PAGE)[15]. The proteins were transferred to a PVDF (Hybond™ P, GE Healthcare) membrane which was incubated with the corresponding primary antibody at the appropriate dilution: against OPG (sc-8468, Santa Cruz Biotechnology, 1:1,000); against α-actin (CP-47, Calbiochem, 1:1,000 dilution) and against GAPDH (scycealdehyde-3-phosphate dehydrogenase) (sc25778, Santa Cruz Biotechnology, 1:5,000). Peroxidase-linked secondary antibodies were specific for each primary antibody (sc-2023, Santa Cruz Biotechnology; and 401215, Calbiochem). Finally, the detection was carried out by the ECL Western Blotting Detection kit (Amersham Biosciences). The development was carried out using the Chemidoc XRS+ kit (Bio-Rad). The determination of the intensity of the bands obtained in the Western blot was performed with the Image Lab software (Bio-Rad).

Statistic analysis
Statistical analysis was carried out using SPSS software for Windows 17.0 (SPSS Inc) and significant differences were considered with p less than 0.05. Results were expressed as mean ± standard deviation. Differences in alkaline phosphatase activity and gene and protein expression between the different culture conditions were assessed using Student's t test after checking the normality of the variables. In the case of the identification of proteins with the Mascot search program, the assumption of the variables' normality has hindered assessing the normality or not of the identified proteins.

Results

Changes in the phenotypic level to the osteoblast-like cell were analyzed in the VSMC after being maintained in culture in the presence or absence of 10^{-7} M calcitriol for 10 days. A significant increase in alkaline phosphatase activity was observed twice in cells exposed to calcitriol (Figure 1A). In addition, a decrease in α-actin in cells exposed to calcitriol was observed by immunocytochemistry, confirming the loss of muscle phenotype with calcitriol at high doses (Figure 1B).

The qPCR study of muscle and bone genes showed that exposure to a high dose of calcitriol significantly decreased α-actin gene expression by 35%, whereas for elastin suppression was almost total (99%) (Figures 2A and 2B). In the case of typically osseous genes, exposure to calcitriol significantly increased (13 fold) the gene expression of osteocalcin, this increase being smaller, but also significant, in the case of Runx2 (2.5 fold) (Figures 2C and 2D).

In order to compare the spectrum of differentially expressed proteins by VSMC exposure to 10^{-7} M calcitriol for 10 days (10^{-7} M CTR group) with respect to VSMC in the absence of calcitriol (control group), proteomic analysis was carried out. There were 334 spots located on each of the 6 gels stained with silver nitrate (3 Control and 3 CTR 10^{-7} M), of which 22 presented significant differences in expression ($p < 0.05$). Of these, Mascot 10 spots were identified by the search engine that are shown in figure 3.

Of the 10 proteins that were identified (Figure 3 and Table 1), we should highlight the increase in expression in 6 of them, among which is the mitochondrial superoxide dismutase, a marker of oxidative stress, which experienced a greater increase. The other proteins that increased its expression were cytoskeletal or related proteins (glial fibrillary acid and threonine/serine kinase type Ste20), proteins involved in vessel formation (dynamins), membrane proteins (ceramide glucosyltransferase) and proteins of the inflammasome (pyrins). In contrast, we identified 4 proteins that after their exposure to calcitriol decreased their expression, with the highest decrease in cytoplasmic actin 2. The other muscle marker whose

expression was also diminished was α-actin of aortic smooth muscle. The other two proteins whose expressions were diminished were prolyl-4-hydroxylase, involved in the maturation of collagen fibers, and inactive dipeptidyl peptidase 10 (DPP10), which is part of potassium channels.

To confirm the results obtained by proteomics the protein expression of α-actin, muscle protein, and OPG of bone strain was analyzed by Western blot analysis. Western blot analysis showed that exposure to calcitriol induced a decrease in α-actin protein expression and an increase in OPG (Figure 4).

Discussion

Research into CV has aroused increasing interest due to its association with cardiovascular morbidity and mortality[16]. The effect of vitamin D as a promoter of CV deserves particular attention, since high doses of calcitriol have been associated experimentally with an increase in CV[9,17]. Therefore, the differential expression profile of VSMC proteins subjected to a supra-physiological dose of calcitriol has been analyzed *in vitro*, finding for the first-time proteins that had not been identified in this process of calcification mediated by calcitriol.

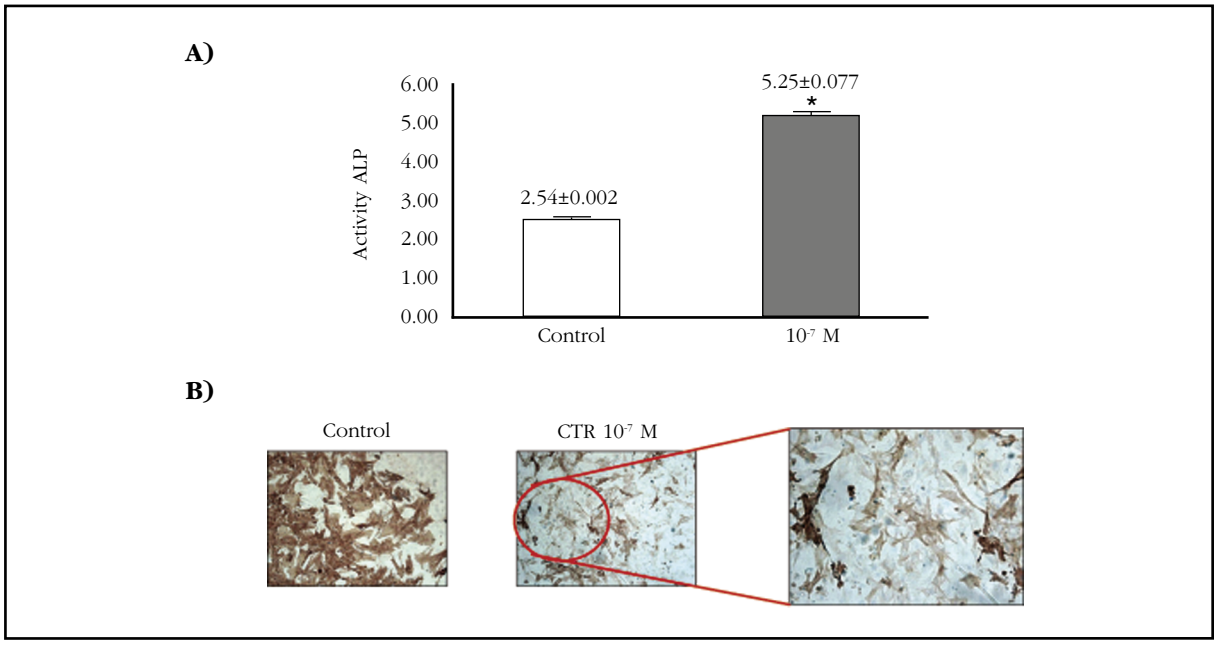
In the present study and according to previous studies[9,18], an increase in alkaline phosphatase activity and a decrease in α-actin protein were observed in cells exposed to calcitriol. This effect can be explained by the increased expression of the transcription factor Runx2, which plays a decisive role in the phenotypic change associated with CV[19]. An increase in the transcription of the osteocalcin bone gene in response to calcitriol and an increase in the protein expression of OPG, both typically bone, have also been observed[20]. In fact, administration of high doses of calcitriol, similar to those used in our study[9,21], has been associated with CV in both nephrectomized rats and in rats with normal renal function[10,21]. In contrast, low doses of calcitriol and its analogs do not induce CV, and may even have a protective effect on its development[22,23].

Parallel to the increase in the expression of bone factors, not only the decrease in α-actin protein levels, but also a decrease in the gene expression of both α-actin and elastin, both muscle markers, have been observed. Since VCAMs show high plasticity[24], VC promoters can induce a decrease in the expression of muscle genes and lead to a differentiation into the bone phenotype, accompanied by an increase in factors that promote calcification[25].

After mass spectrometric analysis of the protein extracts obtained, 10 proteins were identified. Depending on where they exert their function, the identified proteins were classified as: cytoskeleton proteins, involved in the formation of vesicles, membrane proteins, extracellular matrix proteins, inflammatory proteins and proteins related to oxidative stress. The identified proteins were mostly distributed in the cytoplasm, but also in other intracellular organelles such as the endoplasmic reticulum (ER), Golgi apparatus and mitochondria.

Of the differentially expressed proteins identified by LC-MS/MS, six showed increased expression after treatment with calcitriol compared to the control. The one with the greatest overexpression was superoxide dismutase or mitochondrial SOD, which is an oxide-reductase and one of the most important antioxidant enzymes. Such increase

Figure 1. A) Alkaline phosphatase activity (ALP), measured as nmol of p-nitrophenol phosphate/µg protein hydrolyzed per minute, in VCMV exposed to calcitriol for 10 days. B) Immunocytochemistry of α-actin in VSMC in absence (control) or presence of 10^{-7} M calcitriol (10^{-7} M CTR) for 10 days. The red circle is depicted at higher magnification on the right to see the difference in staining. * P<0.05 with respect to the control



could represent a compensatory mechanism to counteract the damage induced by the increase of reactive oxygen species in the calcification process[26-28].

Another protein that is observed to be increased in the VCMVs exposed to calcitriol is the glial fibrillary acidic protein, one of the fibrous proteins that form the intermediate filaments of the intracellular cytoskeleton[29]. This protein is found in certain cells closely related to filaments of vimentin, desmin and periferin, which are involved in the structure and function of the cytoskeleton. There are no data linking the glial fibrillary acidic protein with the CV, but its increase could also represent a compensatory mechanism to avoid the disorganization of the cytoskeleton that occurs in the CV process.

Figure 2. Relative levels of mRNA of A) α-actin, B) elastin, C) osteocalcin and D) Runx2 in VSMC in absence (control) or presence of 10^{-7} M calcitriol (10^{-7} M CTR) for 10 days. *P<0.05 vs control

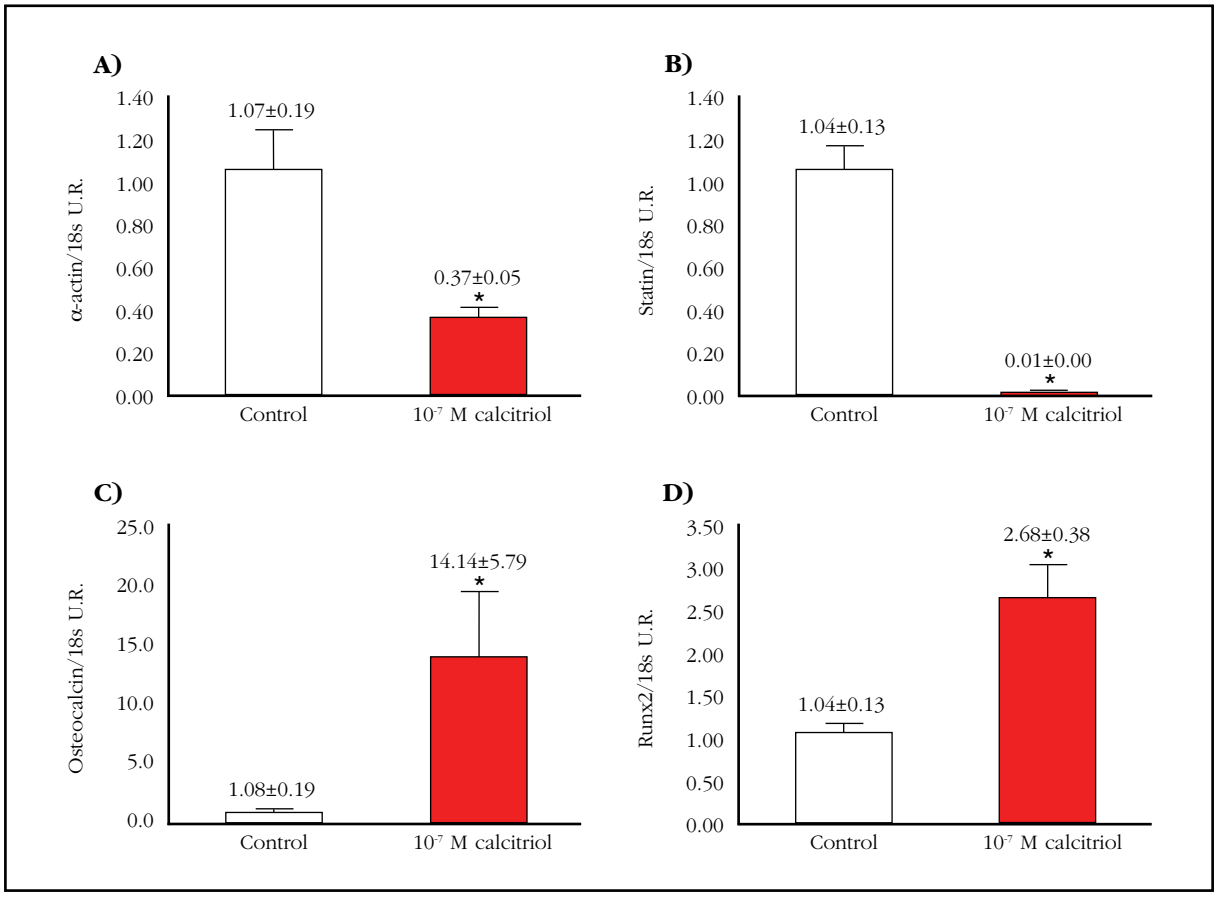


Figure 3. Two-dimensional gels of VSMC protein extracts cultured in absence (control) or presence of 10^{-7} M calcitriol (10^{-7} M CTR) for 10 days. Those proteins expressed differentially with respect to the control cells are indicated: in red those that show increase and in green those that descend

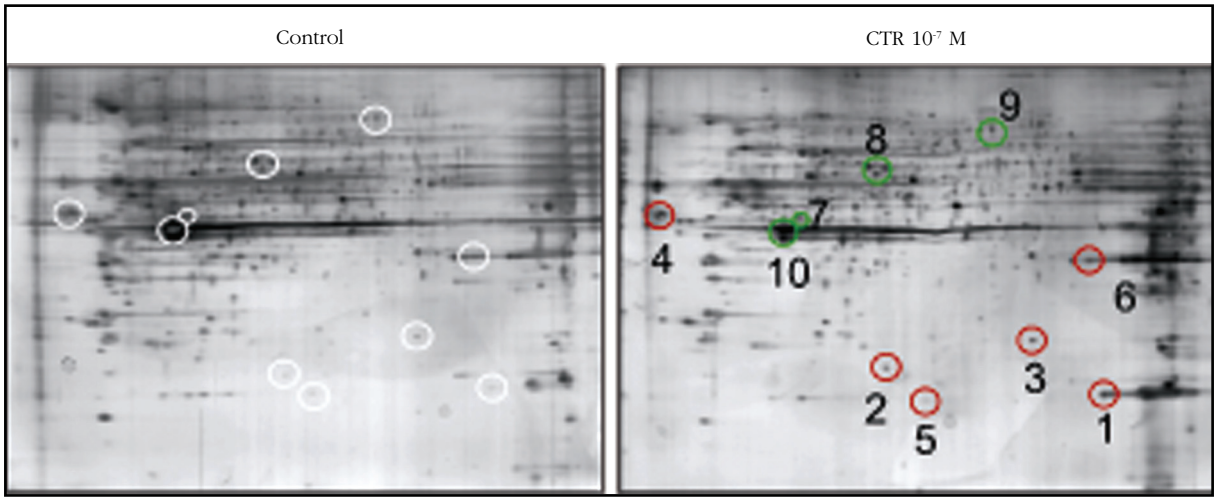


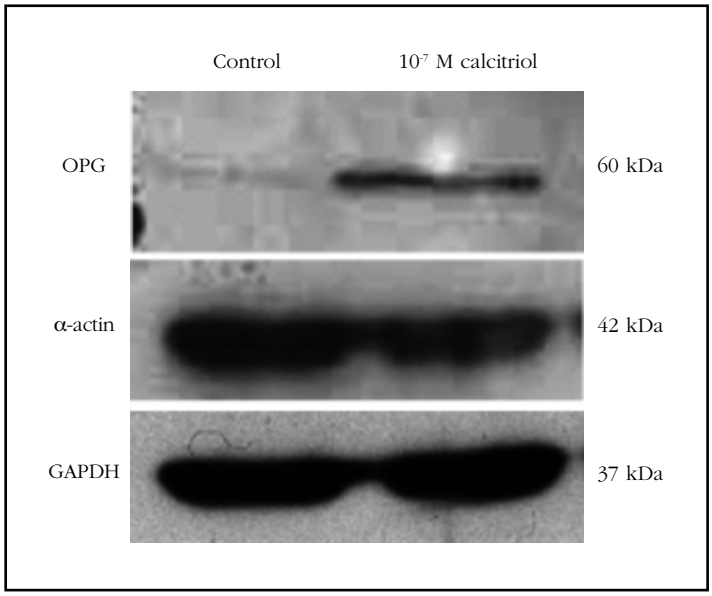
Table 1. List of proteins identified by the Mascot searcher differentially expressed in VSMC cultured in absence (control) or presence of 10^{-7} M calcitriol (10^{-7} M CTR) for 10 days

Name of the protein	Nº acc.	Nº peptides	Control	CTR 10^{-7} M	Exchange rate CTR/ Control	Value p
1: Mitochondrial superoxide dismutase [Mn]	P07895	2	5.5±5.8	15.5±4.2	2.82	0.027
2: Glial fibrillary acidic protein	Q9UFD0	2	5.1±2.2	13.2±0.8	2.59	0.006
3: Protein type dinamine	O00429	2	6.9±3.5	16.2±6.5	2.35	0.024
4: Serine/threonine kinase type Ste20	Q9H2G2	2	23.4±9.3	54.3±9.4	2.32	0.008
5: Ceramide glycosyltransferase	Q16739	3	7.6±1.1	14.9±6.6	1.96	0.029
6: Pyridine	O15553	1	2.8±4.2	4.5±7.3	1.61	0.041
7: α-actin aortic smooth muscle	P62738	1	14.1±1.4	6.2±2.1	0.44	0.004
8: α1 subunit of prolyl-4-hydroxylase	P54001	2	23.2±8.1	9.2±2.7	0.39	0.041
9: Dipeptidyl peptidase 10 inactive	Q8N608	2	33.7±8.1	12.9±8.8	0.38	0.019
10: Cytoplasmic actin 2	P63259	8	763±33	208±11	0.27	0.004

The accession numbers of Swissprot (Nº acc.) And the number of peptides identified by mass spectrometry (Nº peptides) are shown. The average intensity of the spots ± standard deviation of the 3 control gels, the 3 gels CTR 10^{-7} M and the exchange between them (CTR/Control) is represented. P calculated by Student t for independent variables.

In the present study, we also observed an increase in dynamin, which is a GTPase responsible for endocytosis in eukaryotic cells. Dynamins are mainly involved in the excision of newly formed vesicles from the membrane of a cell compartment. This fact could involve them in a common phenomenon and inducer of CV as is the appearance of matrix vesicles, which are formed from cells where mineralization originates or are the result of the process of cellular apoptosis (apoptotic bodies)[30]. In fact, Kashiwakura et al. have observed that dynamin is capable of at least partially regulating apoptosis induced by oxidized low-density lipoproteins by regulating its endocytosis[31]. A recent article has implicated them as a mediator of oxidative stress in cardiomyocytes, helping to slow the production of reactive oxygen species and apoptosis[32].

Figure 4. Overexpression of osteoprotegerin (OPG) and decrease of α-actin by Western blot in VSMC exposed to 10^{-7} M calcitriol for 10 days



Another group of proteins that increased their expression in VSMC by their exposure to calcitriol was the Ste20 threonine/serine kinase. These enzymes are involved in the orientation and organization of spindle microtubules during mitosis[33]. It is known that this protein is a transcriptional regulator of the poloid kinase Plk1 in smooth muscle[34]. A recent study has observed an increase in the expression of this protein in aortas of elderly individuals, suggesting that it promotes the instability of microtubules and actin filaments[35].

Glucosylceramide transferase (GlcT-1) also increased its protein expression. This protein is integrated in the metabolism of sphingolipids, intervening in the transfer of a molecule of glucose for the glycosylation of ceramide (acylsphingo-

sine), giving rise to glycolipid compounds called cerebrosides and which are important components of the cell membrane of muscle tissue. Although there are no data in the literature that relate these enzymes to the vascular calcification process, there is a classic work that finds the accumulation of glycosphingolipids in patients who died with atherosclerotic plaque, which could suggest a pathogenic mechanism of vascular tissue alteration[36].

It is possible to emphasize the increase of a protein called pyrin that forms part of the inflammasome. This is a set of cytosolic multi-proteins that allows the activation of proinflammatory caspases which transform the precursor of interleukin-1β (pro-IL-1 β) to the active form (IL-1 β), leading to a powerful inflammatory response[37]. Recently, Wen et al. have described that, for the calcification of VSMC, the inflammatory response is required[38].

Likewise, in agreement with previous published works carried out in *in vitro* models of endothelial dysfunction and proteomics[39], our results showed a decrease in the expression of structural proteins and a deregulation of the cytoskeletal proteins in the VCLCs exposed to calcitriol at supraphysiological doses. Aortic smooth muscle actin and cytoplasmic actin 2 are proteins that play a key role in cell architecture and motility. This could be interpreted as a loss of the VSMC muscle phenotype as a consequence of exposure to high concentrations of calcitriol.

CV is a regulated pathological process that resembles osteogenesis. When the VSMC of the mean are exposed to a calcifying stimulus, they maintain their ability to differentiate into osteoblast or chondrocyte cells, expressing different bone proteins, producing matrix vesicles and components of the extracellular matrix with propensity for mineralization[40].

In our experiments, in addition to components of the extracellular matrix, a decrease in proteins of the rough endoplasmic reticulum (RER) involved in collagen maturation was detected. Prolyl-4-hydroxylase is an enzyme that participates in post-translational hydroxylation of proline proline and whose decrease inhibits the formation of the triple helix. Although there is no data of this enzyme on CV, the absence of prolyl-3-hydroxylase with which it shares the hydroxylation mechanism of proline in the collagen results in hypermineralization of the bone matrix[41].

Finally, a protein that has shown a decrease is inactive dipeptidyl peptidase 10 (DPP10). This protein, which is an important neuronal component of the potassium channels, can act as a chaperone interacting with other important signaling molecules, such as hsp90 and associated proteins, and can modulate apoptosis[42]. Therefore, the decrease observed in our study in cells exposed to calcitriol could be a consequence of the change in the observed cellular phenotype.

The results of this *in vitro* experimental study have the limitations of not being directly applicable to humans, but should warn of the effect that high doses of calcitriol, used as a treatment of secondary hyperparathyroidism in chronic kidney disease, may have on the vascular calcification. Although in the normal population the kidney would be able to eliminate excess calcitriol, mostly derived from treatment with 25-hydroxyvitamin D and its renal and extra-renal conversion to calcitriol, when deterioration of renal function is aggravated by aging should alert clinicians to follow a very strict control, to avoid harmful effects that could have a high calcitriol, increasing hypercalcemia and hyper-phosphoremia and its subsequent deposition in vascular tissue.

Identification of protein spots is limited by the quantity and quality (low concentration of salts, nucleic acids, lipids, etc.) of the protein extract obtained, which depends directly on the efficiency of the extraction method[43]. In our work, in addition to the proteins described above identified with a high reliability, according to the score obtained in the different databases of protein identification, other proteins were also identified with a low score that could be explained considering different reasons[44]. On the one hand, the concentration of some molecules may be much lower than others in the cut spots. On the other hand, some proteins may have undergone post-translational modifications or proteolysis, which may alter the availability of the peptides for identification. Finally, it should be taken into account that the characterization of the protein profile of the cells has been carried out in a calcification model that lasted up to 10 days. It is possible that, in models where calcification is established in periods of up to 3 weeks, the levels of some proteins could be higher and greater differences observed.

Therefore, and as a summary, we can affirm that we have objectified a decrease in vascular phenotype and an increase of bone in VSMC subjected to a supra-physiological dose of calcitriol. These phenotypic changes give rise to a differential protein profile, with increased expression of proteins involved in free radical scavenging and forming part of the cytoskeleton as a possible compensatory mechanism to the calcification process. It was also observed an increase in proteins involved in the formation of matrix vesicles, as well as in an increase in the inflammatory response, both processes inherent to vascular calcification. The loss of muscle phenotype was represented by declines in the expression of typically muscle proteins. These results should be ratified in animal models with a view to their clinical utility in the prevention of vascular calcifications in the general population.

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

Acknowledgments: This paper has been made possible thanks to the funding obtained by the FEIOMM grant for basic research 2010. This work

has also been partially funded with the help of the National R & D & I Plan 2008-2011, State Plan for R & I 2013-2016, Carlos III Health Institute (ISCIII) – European Regional Development Fund (PI13/00014), Science, Technology and Innovation Plan 2013-2017 of the Principality of Asturias (GRUPIN14-028), Foundation for Development in Asturias Institute for Applied Scientific Research and Technology (FICYT), Reina Sofía Institute for Nephrology Research, Renal Íñigo Álvarez de Toledo Foundation, RETIC RedInRen of ISCIII – European Regional Development Fund (RD06/0016/1013, RD12/0021/1023 and RD16/0009/0017), by the Sociedad Asturiana Fomento Metabólicas Investigaciones.

Bibliography

1. Giachelli CM. Vascular calcification mechanisms. J Am Soc Nephrol. 2004;15:2959-64.
2. Naves M, Rodriguez-Garcia M, Diaz-Lopez JB, Gomez-Alonso C, Cannata-Andia JB. Progression of vascular calcifications is associated with greater bone loss and increased bone fractures. Osteoporos Int. 2008;19:1161-6.
3. Szulc P, Kiel DP, Delmas PD. Calcifications in the abdominal aorta predict fractures in men: MINOS study. J Bone Miner Res. 2008;23:95-102.
4. Rodriguez Garcia M, Naves Diaz M, Cannata Andia JB. Bone metabolism, vascular calcifications and mortality: associations beyond mere coincidence. J Nephrol. 2005;4:458-63.
5. Schinke T, Karsenty G. Vascular calcification–a passive process in need of inhibitors. Nephrol Dial Transplant. 2000;15:1272-4.
6. Hollick, MF. Resurrection of vitamin D deficiency and rickets. J Clin Invest. 2006;116:2062-72.
7. Cannata-Andia JB, Gomez Alonso C. Vitamin D deficiency: a neglected aspect of disturbed calcium metabolism in renal failure. Nephrol Dial Transplant. 2002;17:1875-8.
8. Inoue T, Kawashima H. 1,25-Dihydroxyvitamin D3 stimulates 45Ca2+-uptake by cultured vascular smooth muscle cells derived from rat aorta. Biochem Biophys Res Commun. 1988;152:1388-94.
9. Jono S, Nishizawa Y, Shioi A, Morii H. 1,25-Dihydroxyvitamin D3 increases in vitro vascular calcification by modulating secretion of endogenous parathyroid hormone-related peptide. Circulation. 1998;98:1302-6.
10. Bas A, Lopez I, Perez J, Rodriguez M, Aguilera-Tejero E. Reversibility of calcitriol-induced medial artery calcification in rats with intact renal function. J Bone Miner Res. 2006;21:484-90.
11. Mizobuchi M, Finch JL, Martin DR, Slatopolsky E. Differential effects of vitamin D receptor activators on vascular calcification in uremic rats. Kidney Int. 2007;72:709-15.
12. Panizo S, Cardus A, Encinas M, Parisi E, Valcheva P, López-Ongil S, et al. RANKL increases vascular smooth muscle cell calcification through a RANK-BMP4-dependent pathway. Circ Res. 2009;104:1041-8.
13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method Methods. 2001;25:402-8.
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.
15. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1990;227:680-5.
16. Blacher J, Guerin AP, Pannier B, Marchais SJ, London GM. Arterial calcifications, arterial stiffness, and cardiovascular risk in end-stage renal disease. Hypertension. 2001;38:938-42.
17. Rebsamen MC, Sun J, Norman AW, Liao JK. 1alpha,25-dihydroxyvitamin D3 induces vascular smooth muscle cell migration via activation of phosphatidylinositol 3-kinase. Circ Res. 2000;91:17-24.
18. Shalhoub V, Shatzen EM, Ward SC, Young JI, Boedigheimer M, Twehues L, et al. Chondro/osteoblastic and cardiovascular gene modulation in human artery smooth muscle cells that calcify in the presence of phosphate and calcitriol or paricalcitol. J Cell Biochem. 2010;111;911-21.
19. Sun Y, Byon CH, Yuan K, Chen J, Mao X, Heath JM, et al. Smooth muscle cell-specific runx2 deficiency inhibits vascular calcification. Circ Res. 2012;111:543-52.
20. Gori F, Hofbauer LC, Dunstan CR, Spelsberg TC, Khosla S, Riggs BL. The expression of osteoprotegerin and RANK ligand and the support of osteoclast formation by stromal-osteoblast lineage cells is developmentally regulated. Endocrinology. 2000;141:4768-76.
21. Wu-Wong JR, Noonan W, Ma J, Dixon D, Nakane M, Bolin AL, et al. Role of phosphorus and vitamin D analogs in the pathogenesis of vascular calcification. J Pharmacol Exp Ther. 2006;318:90-8.
22. Li X, Speer MY, Yang H, Bergen J. Giachelli CM. Vitamin D receptor activators induce an anticalcific paracrine program in macrophages: requirement of osteopontin. Arterioscler Thromb Vasc Biol. 2010;30:321-6.
23. Aoshima Y, Mizobuchi M, Ogata H, Kumata C, Nakazawa A, Kondo F, et al. Vitamin D receptor activators inhibit vascular smooth muscle cell mineralization induced by phosphate and TNF-alpha. Nephrol Dial Transplant. 2012;27:1800-6.
24. Katoh Y, Periasamy M. Growth and differentiation of smooth muscle cells during vascular development. Trends Cardiovasc Med. 1996;6:100-6.
25. Tukaj C, Kubasik-Juraniec J, Kraszpulski M. Morphological changes of aortal smooth muscle cells exposed to calcitriol in culture. Med Sci Monit. 2000;6:668-74.
26. Byon CH, Javed A, Dai Q, Kappes JC, Clemens TL, Darley-Usmar VM, et al. Oxidative stress induces vascular calcification through modulation of the osteogenic transcription factor Runx2 by AKT signaling. J Biol Chem. 2008;283:15319-27.
27. Sutra T, Morena M, Bargnoux AS, Caporiccio B, Canaud B, Cristol JP. Superoxide production: a procalcifying cell signalling event in osteoblastic differentiation of vascular smooth muscle cells exposed to calcification media. Free Radic Res. 2008;42:789-97.
28. Roman-Garcia P, Barrio-Vazquez S, Fernandez-Martin JL, Ruiz-Torres MP, Cannata-Andia, JB. Natural antioxidants and vascular calcification: a possible benefit. J Nephrol. 2011;24:669-72.
29. Lewis GP, Matsumoto B, Fisher SK. Changes in the organization and expression of cytoskeletal proteins during retinal degeneration induced by retinal detachment. Invest Ophthalmol Vis Sci. 1995;36:2404-16.
30. Kapustin AN, Chatrou MLL, Drozdov I, Zheng Y, Davidson SM, Soong D, et al. Vascular smooth muscle cell calcification is mediated by regulated exosome secretion. Circ Res. 2015;116:1312-23.
31. Kashiwakura Y, Watanabe M, Kusumi N, Sumiyoshi K, Nasu Y, Yamada H, et al. Dynamin-2 regulates oxidized low-density lipoprotein–induced apoptosis of vascular smooth muscle cell. Circulation. 2004;110:3329-34.
32. Gao D, Yang J, Wu Y, Wang Q, Wang Q, Lai EY, et al. Targeting dynamin 2 as a novel pathway to inhibit cardiomyocyte apoptosis following oxidative stress. Cell Physiol Biochem. 2016;39:2121-34.
33. Zhapparova ON, Fokin AI, Vorobyeva NE, Bryantseva SA, Nadezhdina ES. Ste20-like protein kinase SLK (LOSK) regulates microtubule organization by targeting dynactin to the centrosome. Mol Biol Cell. 2013;24,3205-14.
34. Li J, Wang R, Gannon OJ, Rezey AC, Jiang S, Gerlach BD, et al. Polo-like kinase 1 regulates vimentin phosphory-

lation at Ser-56 and contraction in smooth muscle. J Biol Chem. 2016;291:23693-703.
35. Fu Z, Wang M, Everett A, Lakatta E, Van Eyk J. Can proteomics yield insight into aging aorta? Proteomics Clin Appl. 2013;7 477-89.
36. Chatterjee SB, Dey S, Shi WY, Thomas K, Hutchins GM. Accumulation of glycosphingolipids in human atherosclerotic plaque and unaffected aorta tissues. Glycobiology. 1997;7:57-65.
37. Drenth JPH, van der Meer JWM. The Inflammasome - a linebacker of innate defense. N Engl J Med. 2006;355:730-2.
38. Wen C, Yang X, Yan Z, Zhao M, Yue X, Cheng X, et al. Nalp3 inflammasome is activated and required for vascular smooth muscle cell calcification. Int J Cardiol. 2013;168:2242-7.
39. Carbó C, Arderiu G, Escolar G, Fusté B, Cases A, Carrascal M, et al. Differential expression of proteins from cultured endothelial cells exposed to uremic versus normal serum. Am J Kidney Dis. 2008;51:603-12.
40. Neven E, Persy V, Dauwe S, De Schutter T, De Broe ME, D'Haese PC. Chondrocyte rather than osteoblast conversion of vascular cells underlies medial calcification in uremic rats. Arterioscler Thromb Vasc Biol. 2010;30:1741-50.
41. Fratzl-Zelman N, Bächinger HP, Vranka JA, Roschger P, Klaushofer K, Rauch F. Bone matrix hypermineralization in prolyl-3 hydroxylase 1 deficient mice. Bone. 2016;85:15-22.
42. Tsaprouni L, Ito K, Cookson WO, Moffatt MF, Barnes PJ, Adcock IM. Functional genomics of DPP10. Protein characterization and its association with asthma and COPD. Am J Respir Crit Care Med. 2007;175:A387.
43. Gil-Dones F, Martín-Rojas T, López-Almodóvar LF, Juárez-Tosina R, de la Cuesta F, Álvarez-Llamas G, et al. Obtención de un protocolo óptimo para el análisis proteómico de válvulas aórticas humanas sanas y estenóticas. Rev Esp Cardiol. 2010;63:46-53.
44. Bagnato C, Thumar J, Mayya V, Hwang SI, Zebroski H, Claffey KP, et al. Proteomics analysis of human coronary atherosclerotic plaque: a feasibility study of direct tissue proteomics by liquid chromatography and tandem mass spectrometry. Mol Cell Proteomics. 2007;6:1088-102.

Panizo García S, Carrillo López N, Martínez Arias L, Román García P, Cannata Andía JB, Naves Díaz M

Servicio de Metabolismo Óseo y Mineral - Instituto de Investigación Sanitaria del Principado de Asturias - Red de Investigación Renal (REDinREN) del Instituto de Salud Carlos III (ISCIII) - Universidad de Oviedo - Hospital Universitario Central de Asturias - Oviedo (España)

The effect of oxidative stress on vascular calcification through microRNA-377

DOI: http://dx.doi.org/10.4321/S1889-836X2017000400006

Correspondence: Manuel Naves Díaz - Servicio de Metabolismo Óseo y Mineral - Hospital Universitario Central de Asturias - Avenida de Roma, s/n - 33011 Oviedo (Spain)
e-mail: manuel@hca.es

Date of receipt: 23/06/2017
Date of acceptance: 22/07/2017

Work submitted as a grant for the FEIOMM scholarship received to attend the 33rd Congress of the ASBMR (San Diego, 2011).

Summary

Introduction: Oxidative stress has been implicated in the development and progression of vascular calcification (VC). However, this causal association remains a matter of controversy.

Objective: To analyze in an experimental model of chronic renal failure (CRF), the effect of oxidative stress on the development and progression of the VC, assessing the implication of microRNA-377 (miR-377).

Material and methods: Two groups of Wistar rats with CRF were studied. Group 1 received normal diet in phosphorus (CRF+NP). Group 2 received a high phosphorus (CRF+HP) diet. A group of Sham rats was included. After 20 weeks, the rats were sacrificed.

Results: Serum phosphorus and parathormone did not increase in the CRF+HP group compared to CRF+NP, but fibroblast growth factor 23 (FGF23) levels significantly increased. In the CRF+NP group, aortic calcium content increased three-fold over the Sham group, a 17-fold increase in the CRF+HP group, where the bone mineral density in the proximal tibia decreased significantly. In the CRF+NP group, the expression of miR-377 decreased by 65%, with no additional effect detected of the diet with high phosphorus content. In the CRF+NP group, the protein expression of mitochondrial superoxide dismutase 2 (SOD-2) increased 3-fold, and in the CRF+HP group it increased up to 6-fold.

Conclusions: CRF, with or without high phosphorus dietary content, triggered the descent of miR-377. Excess phosphorus increased SOD-2 as a compensatory mechanism to curb oxidative stress and vascular damage. Controlling phosphorus content in the diet when the renal impairment function is compromised will reduce the vascular damage produced due oxidative stress, among other factors.

Key words: *vascular calcification, miR-377, SOD-2, oxidative stress, BMD.*

Introduction

Vascular calcification (VC) is one of the most common disorders in aging, but especially in patients with chronic kidney disease (CKD). The increase of reactive oxygen species (ROS) in response to overloads in phosphorus (P) which occurs during VC contributes to the phenotypic dedifferentiation of vascular smooth muscle cells (VSMC) from a properly muscular (contractile) phenotype to an osteoblastic phenotype[1-4]. In particular, hydrogen peroxide is considered one of the most common ROS, capable of inducing the expression of the osteoblastic transcription factor Cbfa1/RUNX2 and inducing calcification[5,6].

MicroRNAs (miRs) are small, non-coding single stranded RNAs (~22 nucleotides) that mediate the posttranscriptional silencing of genes by binding through complementarity of bases to the 3 'UTR region of the target mRNAs. The miRs are involved in crucial biological processes, including cell proliferation, differentiation and tissue development[7,8]. Recent studies have shown that several miRs are important regulators of VSMC differentiation into cells resembling osteoblasts and, therefore, vascular calcification[9-12].

The miR-377 appears as an important aging regulator by regulating, among others, superoxide dismutase 2 enzyme (SOD-2)[13,14]. SOD is the body's main antioxidant, which catalyzes the conversion of superoxide radicals to hydrogen peroxide. SOD-2 corresponds to the mitochondrial form of the enzyme. Several articles have considered the role of oxidative stress as an inducer of VC, so in the present study we propose to analyze the possible role of miR-377 as regulator of aortic mineralization in an in vivo model.

Material and methods

Model of vascular calcification

Our research protocol was approved by Oviedo University's Ethics Committee for Animal Experimentation. The study was carried out using male Wistar rats (n=10) at 4 months of age (350-400 g) who underwent chronic renal failure (CRF) (7/8) in a single surgical procedure, after isoflurane inhalation anesthesia. Complete nephrectomy of the right kidney and then subtotal left kidney nephrectomy were carried out by lateral incision and in the upper area. With this procedure, approximately one-quarter of the renal mass is conserved. CRF rats were divided into two groups: one, NP, fed a standard rodent diet with a normal P content (0.6%) and 0.6% calcium (Ca), and a protein content of 20 % (Panlab, Barcelona, Spain); The other, HP group, was fed a diet high in P (0.9%), 0.6% Ca, and a protein content of 20% (Panlab, Barcelona, Spain). The study lasted 20 weeks in order to induce vascular calcifications. A Sham group (n=5) was also included and followed up to week 20. Twenty-four hours before slaughter, the rats were housed in metabolic cages to obtain urine samples in each case receiving the same diet and water ad libitum. They were sacrificed using CO_2 anesthesia, and serum samples were taken for analysis. The descending abdominal aorta was removed to the iliac bifurcation from each rat and divided into three portions: the first fragment closest to the aortic arch to determine the Ca content, the second fragment was used for RNA extraction and proteins, and the third fragment was included in paraffin for future studies.

Two tibias were removed at the time of sacrifice. The left was preserved in alcohol to measure bone mineral density (BMD). The remaining tibia was frozen at -80°C.

Biochemical markers

Serum Ca, P and creatinine and urine creatinine levels were measured using a Hitachi 717 multichannel automatic analyzer (Boehringer Mannheim, Berlin, Germany). Serum parathormone (PTH) was measured by ELISA (Immutopics, San Juan Capristano, USA) following the manufacturer's protocols. Fibroblast growth factor 23 (FGF23) was determined by an ELISA kit (Kainos Laboratories, Japan).

Bone densitometry

BMD was measured in the tibia at three levels: proximal octave, seventh/eighth distal and total tibia, with a Hologic QDR-1000 dual-energy digital radiological densitometer (Hologic, Bedford, USA) equipped with a specific program for small animals.

Analysis of aortic calcification

Abdominal aortic calcification of the rats was analyzed for total Ca content. To determine this, a fragment of the abdominal aorta (closest to the aortic arch) was homogenized with an Ultra-Turrax (OmniHT) in 0.6 N HCl. After stirring at 4°C for 24 hours the samples were centrifuged. Ca content was determined in the supernatant by the o-cresolphthalein complexone method (Sigma-Aldrich, St. Louis, USA), and the cell pellet was resuspended in lysis buffer (125 mM Tris and 2% SDS, PH 6.8) for protein extraction and quantification by the Lowry assay (Bio-Rad, Hercules, USA). The Ca content was normalized by expressing as µg Ca per mg of protein.

Study of gene expression

RNA extraction was carried out by the guanidinium-phenol-chloroform thiocyanate method. The DNA copy (cDNA) was synthesized using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). Gene expression of miR-377 was analyzed by real-time PCR (qPCR) in the Stratagene Mx3005P QPCR System (Agilent Technologies, Santa Clara, USA). An assay on-demand designed by Applied Biosystems employing specific oligos and fluorescent TaqMan probes was used for the PCR. For quantification and normalization, nuclear RNA U6 expression was used.

Western Blot

After transfer, the membranes were incubated for

12 hours with anti-SOD-2 (1:1000, Cell Signaling Technology, Danvers, USA) and anti-GAPDH (1:5000, Santa Cruz Biotechnology, Dallas, USA.). Binding of the secondary antibody was detected with the Western Blot detection kit ECL Advance (Amersham Bioscience, Buckinghamshire, UK) and the VersaDoc 4000 imaging system (Bio-Rad).

Statistic analysis

The SPSS 17.0 program was used for the statistical analysis of the results. In the case of variables with normal distribution, the comparison of the treatment groups was carried out using the analysis of variance (ANOVA) with the Bonferroni test. In the case of variables with non-normal distribution, the Kruskal-Wallis test was used.

Results

Nephrectomy decreased creatinine clearance, an effect that did not worsen in animals fed the high P diet (Table 1). There was a discrete but not significant increase in serum P in the nephrectomized animals, which became more noticeable in those who received the high P diet. However, small increases in serum P were associated with more significant increases in serum levels of FGF23. Serum FGF23 levels increased twice with nephrectomy, while serum P increased by less than 50%. Similarly, in animals that received the diet high in P (CRF+HP), FGF23 levels increased twice as much as those in the normal diet (CRF+NP), with an increase in serum P less than 25%.

Nephrectomy had no effect on BMD changes in tibia, although CRF+HP in BMD in proximal tibia decreased significantly with respect to the CRF+NP group and the Sham group (Table 1).

In the CRF+NP group, Ca content in the aorta was increased 3-fold with respect to the Sham group. The increase with respect to the Sham group was increased up to 17 times in the CRF+HP group. The miR-377 expression decreased by 65% with nephrectomy, with no additional effect observed with the high-P diet. In fact, creatinine clearance was the biochemical parameter that was most strongly associated with the expression of this miR (R=0.83, p=0.001). In contrast, serum P was not associated with miR-377 expression (r=-0.302; p=0.34).

Protein expression of SOD-2 was clearly increased with nephrectomy (more than 3-fold), an effect that became more marked in nephrectomized animals with the high-P diet (more than 6-fold).

Discussion

In this study, a decrease in aortic expression of miR-377 with nephrectomy was observed in parallel with the increase in SOD-2 protein expression as a possible compensatory mechanism to eliminate superoxide free radicals produced as a consequence of oxidative stress generated by nephrectomy.

To date, several hundred miRs have been identified in the human genome by proposing that at least 50% of the genes coding for proteins are regulated by miRs[15,16].

The increase in the protein expression of SOD-2, as a possible compensatory effect to curb oxidative stress and cellular apoptosis in the mitochondria[4,5,17], has been observed in our group's other in vitro studies exposing VSMC to calcifying media. In fact, in a recent article we observed using two-dimensional gel-free proteomics techniques that, in the presence of a calcifying stimulus with high doses of calcitriol, the expression of this protein increases 3 fold with respect to the cells cultured with a control medium[18]. Another in vitro study in VSMC subjected to a calcification stimulus by excess Ca and P shows an increase in the protein expression of SOD-2 by Western blot[6].

However, unlike what we have reported in our work, most studies in the literature show SOD-2 decreases in the presence of increased oxidative stress. In fact, in a recent study in rats with CRF, it has been found that the administration of a high P diet supplemented with calcitriol induces a decrease in the protein expression of SOD-2 at the aortic level[19]. Other studies in other tissue models (renal podocytes and mesangial cells) have found that, in the case of stimuli inducing oxidative stress, there is a decrease in SOD-2 with increases in miR-377[20]. These increases have also been implicated with the increase of cellular senescence[14,21].

SOD-2 is also referred to as mitochondrial superoxide dismutase or manganese superoxide dismutase (MnSOD). It is responsible for the reduction of ROS toxic to mitochondria.

The fact that in our case an increase in SOD-2 is observed, in the cell's attempt to defend itself against oxidative stress, accompanied by a decrease in miR-377 expression, may be attributed to different experimental conditions from the rest of the studies, as well as the time frame of renal damage, which in our case is always very long term. What does concur with all published reports is the opposite effect between miR-377 and SOD-2[14,20,21]. That is, increases in SOD-2 lead to decreases in miR-377 expression and vice versa, so the possible usefulness of miR-377 as a biomarker of oxidative stress and vascular damage may be inferred.

Despite using a high P diet, serum P levels were very similar in the nephrectomized animal groups with normal or high P diet. One possible explanation is that 7/8 nephrectomy has not been aggressive enough so a lower grade of nephrectomy may be 5/6 or less. This would indicate maintaining a residual renal function that would avoid aggravating the renal damage caused by P[22]. However, FGF23 notably increases with nephrectomy and also in animals that received the high-P diet. FGF23 is known to be the first biochemical parameter to rise after CRF[23]. FGF23 begins to increase in the plasma of patients with CRD from very early situations, and continues to increase steadily as glomerular filtration declines[24]. During the early stages of CRD, an overload of P would

Table 1. Biochemical and bone metabolism markers in the different treatment groups. BMD values in the three tibial segments analyzed in the different treatment groups

	Sham (n=5)	CRF+NP (n=5)	CRF+HP (n=5)
Creatinine clearance (mL/min)	2.9 ± 2.9	0.8 ± 0.3[a]	1.0 ± 0.5[a]
Calcium (mg/dL)	10.3 ± 0.1	11.6 ± 0.4[a]	10.8 ± 0.6
Phosphorus (mg/dL)	4.1 ± 0.3	5.9 ± 0.8[a]	7.3 ± 4.0
PTH (pg/mL)	3 (1-7)	83 (37-96)[a]	95 (52-610)[a]
FGF23 (pg/mL)	272 ± 140	589 ± 198[a]	1013 ± 40[ab]
Proximal tibia BMD (mg/cm^2)	325 ± 12	327 ± 21	273 ± 26[ab]
Distal tibia BMD (mg/cm^2)	256 ± 15	255 ± 9	234 ± 11
Total tibia BMD (mg/cm^2)	270 ± 12	269 ± 12	243 ± 15

[a]p<0.05 relative to the Sham group; [b]p<0.05 relative to the CRF+NP group.

Figure 1. Ca content in aortas of 7/8 nephrectomy rats fed a diet with normal P content (0.6%) (CRD+NP) and high P content (0.9%) (CRD+HP), sacrificed at 20 weeks. Data represent the mean ± standard deviation. [a]p<0.05 relative to the Sham group; [b]p<0.05 relative to the CRD+NP group

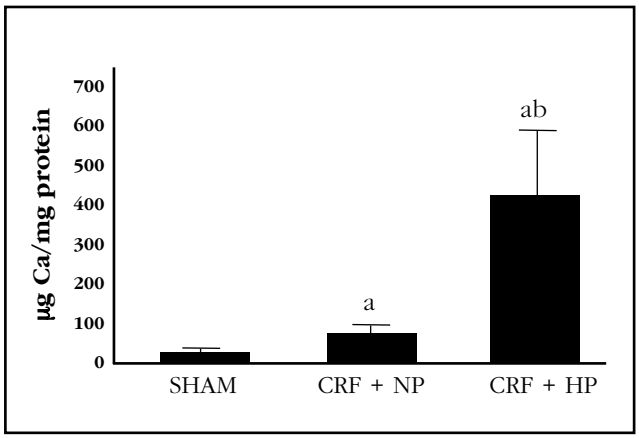
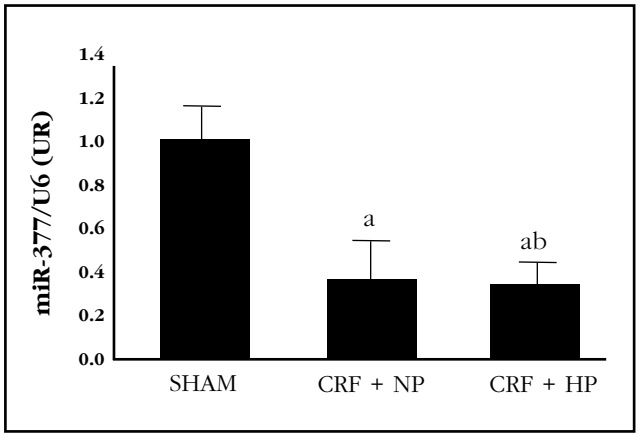


Figure 2. Relative levels of miR-377 in aortas of 7/8 nephrectomy rats fed a diet with normal content in P (0.6%) (CRF+NP) and high P content (0.9%) (CRD+HP), sacrificed at 20 weeks. Data represent the mean ± standard deviation. [a]p<0.05 relative to the Sham group



stimulate the synthesis of FGF23 at the osteocyte level, and would act on the remaining nephrons by increasing the fractional excretion of phosphate to maintain normal phosphatemia[25]. In fact, in our case, increases of less than 25% in serum P were accompanied by much higher FGF23 variations as a mechanism to compensate for the increase in P in order to curb their excess.

The fact that kidney dysfunction obtained by nephrectomy was lower than initially predicted is reflected in the BMD results in the tibia. There were no differences in BMD between Sham animals and nephrectomized animals, despite the time course of CRD (20 weeks)[26]. In the nephrectomized animals that received the high P diet, there was a decrease in BMD in proximal tibia compared to the nephrectomized animals with normal diet in P and in the Sham group. However, other studies in our group, with the same follow-up time, show a more negative effect on cortical bone (BMD in distal tibia) than on the trabecular (BMD in proximal tibia) when the diet administered is rich in P[27]. This effect is accompanied by severe secondary hyperparathyroidism that mainly affects the cortical bone, a result that we did not observe in the present study.

Despite the lower degree of renal dysfunction observed, we detected a relationship between the VC increase and the decrease in BMD, as previously described by our group[28]. The group of animals with CRF and high P diet showed a clear increase in calcium content in the aorta, which was accompanied by the higher losses of BMD in the proximal tibia.

From the results of this study, nephrectomy, independent of serum P levels, is capable of modifying miR-377 expression. The high P content in the diet induced increased

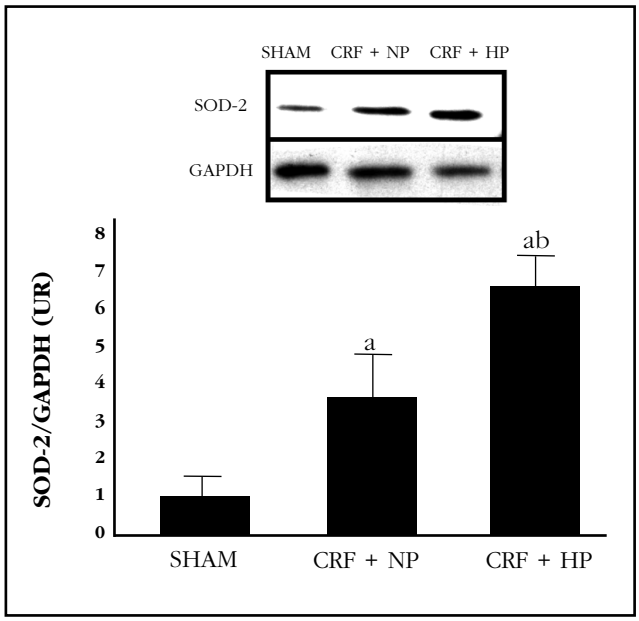
protein expression of SOD-2, probably as a protective mechanism to avoid further vascular damage due to oxidative stress. These results alert us to the desirability of maintaining serum levels of P when renal deterioration is accentuated thus avoiding progressive vascular damage. The use of miR-377 as a biomarker of vascular damage due to oxidative stress requires mechanistic studies, but opens a possible preventive and therapeutic route to the development and progression of vascular lesions in CRD.

Acknowledgments: This research study was made possible thanks to the FEIOMM funding grant awarded Natalia Carrillo López to attend the 33rd Congress of the ASBMR 2011 (San Diego, 2011). The work has also been partially funded with the help of the National Plan for R & D & I 2008-2011, State Plan for R & D & I 2013-2016, Carlos III Health Institute (ISCIII) - European Regional Development Fund/00415), Science, Technology and Innovation Plan 2013-2017 of the Principality of Asturias (GRUPIN14-028), Foundation for the Promotion in Asturias of Applied Scientific Research and Technology (FICYT), Reina Sofía Institute for Nephrological Research, Íñigo Álvarez de Toledo Renal Foundation, RETIC RedInRen of the ISCIII - European Regional Development Fund (RD06/0016/1013, RD12/0021/1023 and RD16/0009/0017), by the Asturian Society for Metabolic Research.

Conflict of interest: The authors hereby declare they have no conflict of interest in this work.

The handling of experimental animals was carried out in accordance with the current legal regulations (European Union Directive 2010/63/EU and Spanish Royal Decree 53/2013 of 1 February).

Figure 3. Protein expression of SOD-2 in the aortas of 7/8 nephrectomy rats fed a diet with normal content in P (0.6%) (CRF+NP) and high P content (0.9%) (CRD+HP), sacrificed at 20 weeks. A) Image of a Western blot representing one aorta of each group; B) graphical representation of the protein expression of SOD-2 in the different groups as mean ± standard deviation. [a]p 0.05 compared to the Sham group; [b]p<0.05 compared to the CRD+NP



Bibliography

1. Xie C, Ritchie RP, Huang H, Zhang J, Chen YE. Smooth muscle cell differentiation in vitro: models and underlying molecular mechanisms. Arterioscler Thromb Vasc Biol. 2011;31:1485-94.
2. House SJ, Potier M, Bisaillon J, Singer HA, Trebak M. The non-excitable smooth muscle: calcium signaling and phenotypic switching during vascular disease. Pflugers Arch. 2008;456:769-85.
3. Shanahan CM. Mechanisms of vascular calcification in CKD-evidence for premature ageing? Nat Rev Nephrol. 2013;9:661-70.
4. Sutra T, Morena M, Bargnoux AS, Caporiccio B, Canaud B, Cristol JP. Superoxide production: a procalcifying cell signalling event in osteoblastic differentiation of vascular smooth muscle cells exposed to calcification media. Free Radic Res. 2008;42:789-97.
5. Byon CH, Javed A, Dai Q, Kappes JC, Clemens TL, Darley-Usmar VM, et al. Oxidative stress induces vascular calcification through modulation of the osteogenic transcription factor Runx2 by AKT signaling. J Biol Chem. 2008;283:15319-27.
6. Roman-Garcia P, Barrio-Vazquez S, Fernandez-Martin JL, Ruiz-Torres MP, Cannata-Andia JB. Natural antioxidants and vascular calcification: a possible benefit. J Nephrol. 2011;24:669-72.
7. Bartel PD. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281-97.
8. Chen K, Rajewsky N. The evolution of gene regulation by transcription factors and microRNAs. Nat Rev Genet. 2007;8:93-103.
9. Balderman JA, Lee HY, Mahoney CE, Handy DE, White K, Annis S, et al. Bone morphogenetic protein-2 decreases microRNA-30b and microRNA-30c to promote vascular smooth muscle cell calcification. J Am Heart Assoc. 2012;1:e003905.
10. Liao XB, Zhang ZY, Yuan K, Liu Y, Feng X, Cui RR, et al. MiR-133a modulates osteogenic differentiation of vascular smooth muscle cells. Endocrinology. 2013;154:3344-52.
11. Rangrez AY, M'Baya-Moutoula E, Metzinger-Le Meuth V, Hénaut L, Djelouat MS, Benchitrit J, et al. Inorganic phosphate accelerates the migration of vascular smooth muscle cells: evidence for the involvement of miR-223. PLoS One. 2012;7:e47807.
12. Panizo S, Naves-Díaz M, Carrillo-López N, Martínez-Arias L, Fernández-Martín JL, Ruiz-Torres MP, et al. MicroRNAs 29b, 133b, and 211 regulate vascular smooth muscle calcification mediated by high phosphorus. J Am Soc Nephrol. 2016;27:824-34.
13. Duan S, Wang Y, Wang H, Wang S, Ji L, Dai D, et al. A novel PCR-based approach to discover miRNA target genes. Int J Med Sci. 2014;11:1270-4.
14. Wang Q, Wang Y, Minto AW, Wang J, Shi Q, Li X, et al. MicroRNA-377 is up-regulated and can lead to increased fibronectin production in diabetic nephropathy. FASEB J. 2008;22:4126–35.
15. Jackson, RJ, Standart N. How do microRNAs regulate gene expression? Sci STKE. 2007;367re1.
16. Faller M, Matsunaga M, Yin S, Loo JA, Guo F. Heme is involved in microRNA processing. Nat Struct Mol Biol. 2007;14:23-9.
17. Chen Y, Cai J, Murphy TJ, Jones DP. Overexpressed human

mitochondrial thioredoxin confers resistance to oxidant-induced apoptosis in human osteosarcoma cells. J Biol Chem. 2002; 277: 33242-8.
18. Carrillo López N, Tuñón LePoultel D, Quirós Caso C, Rodríguez I, Cannata Andía JB, Naves Díaz M. Efecto de dosis suprafisiológicas de calcitriol sobre la expresión proteica de células de músculo liso vascular. Rev Osteoporos Metab Miner. 2017 [Epub ahead of print].
19. Agharazii M, St-Louis R, Gautier-Bastien A, Ung RV, Mokas S, Larivière R, et al. Inflammatory cytokines and reactive oxygen species as mediators of chronic kidney disease-related vascular calcification. Am J Hypertens. 2015;28:746-55.
20. Wang W, Ding XQ, Gu TT, Song L, Li JM, Xue QC, et al. Pterostilbene and allopurinol reduce fructose-induced podocyte oxidative stress and inflammation via microRNA-377. Free Radic Biol Med. 2015;83:214-26.
21. Xie HF, Liu YZ, Du R, Wang B, Chen MT, Zhang YY, et al. miR-377 induces senescence in human skin fibroblasts by targeting DNA methyltransferase 1. Cell Death Dis. 2017;8(3):e2663.
22. Voormolen N, Noordzij M, Grootendorst DC, Beetz I, Sijpkens YW, van Manen JG, et al. High plasma phosphate as a risk factor for decline in renal function and mortality in pre-dialysis patients. Nephrol Dial Transplant. 2007;22: 2909-16.
23. Hasegawa H, Nagano N, Urakawa I, Yamazaki Y, Iijima K, Fujita T, et al. Direct evidence for a causative role of FGF23 in the abnormal renal phosphate handling and vitamin D metabolism in rats with early-stage chronic kidney disease. Kidney Int. 2010;78:975-80.
24. Liu S, Tang W, Zhou J, Stubbs JR, Luo Q, Pi M, et al. Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. J Am Soc Nephrol. 2006;17:1305-15.
25. Lloret MJ, Bover J, DaSilva I, Furlano M, Ruíz-García C, Ayasreh N, et al. Papel del fósforo en la enfermedad renal crónica. Nefrología (Suplemento Extraordinario). 2013;4:2-10.
26. Naves-Díaz M, Carrillo-López N, Rodríguez-Rodríguez A, Braga S, Fernández-Coto T, Lopez-Novoa JM, et al. Differential effects of 17A-estradiol and raloxifene on bone and lipid metabolism in rats with chronic kidney disease and estrogen insufficiency. Bone. 2010;17:766-71.
27. Carrillo-Lopez N, Panizo S, Alonso-Montes C, Román-García P, Rodríguez I, Martínez-Salgado C, et al. Direct inhibition of osteoblastic Wnt pathway by fibroblast growth factor 23 contributes to bone loss in chronic kidney disease. Kidney Int. 2016;90:77-89.
28. Román-García P, Carrillo-López N, Fernández-Martín JL, Naves-Díaz M, Ruiz-Torres MP, Cannata-Andía JB. High phosphorus diet induces vascular calcification, a related decrease in bone mass and changes in the aortic gene expression. Bone. 2010;46:121-8.

Rodríguez Duque JC[1], Núñez Céspedes J[2], Montes Moreno S[3], Mazorra Horts R[3], del Rey Rozas A[4], Olmos Martínez JM[1]

1 Departamento de Medicina Interna - Hospital Universitario Marqués de Valdecilla-IDIVAL (Instituto de Investigación Marqués de Valdecilla) - Universidad de Cantabria - Santander (España)

2 Servicio de Hematología - Hospital Universitario Marqués de Valdecilla-IDIVAL - Universidad de Cantabria - Santander (España)

3 Servicio de Anatomía Patológica - Hospital Universitario Marqués de Valdecilla-IDIVAL - Universidad de Cantabria - Santander (España)

4 Medicina Familiar y Comunitaria - Centro de Salud Puertochico - Santander (España)

Primary bone lymphoma

DOI: http://dx.doi.org/10.4321/S1889-836X2017000400007

Correspondence: José Manuel Olmos Martínez - Dpto. Medicina Interna - Hospital Universitario Marqués de Valdecilla - Avda. Valdecilla, s/n - 39008 Santander (Spain)
e-mail: miromj@humv.es

Date of receipt: 07/06/2017
Date of acceptance: 20/07/2017

Summary

Skeletal involvement in patients with non-Hodgkin's lymphoma (NHL) is not uncommon. It tends to be a late manifestation and usually occurs secondary to lymphomas in advanced stage, with high tumor burden. However, only in a few cases has skeletal involvement been attributed to a primary bone lymphoma and constitutes, therefore, the form of presentation of this disease. We describe the case of a patient with primary B-cell lymphoma of the bone that appeared with vertebral lesions and secondary spinal compression.

Key words: *non-Hodgkin's lymphoma B, primary bone lymphoma, spinal cord compression.*

Introduction

Skeletal involvement in non-Hodgkin's lymphomas (NHL) is not uncommon, although when this occurs it generally presents secondary to advanced lymphomas with high tumor burden. Primary bone lymphoma (PBL) accounts for about 1-5% of malignant bone tumors and accounts for only 5% of extra-nodal lymphomas and less than 1% of lymphomas in general[1-3]. Lesions, which may be single or multiple, are preferentially located in long bones, especially in the femur and, more rarely, in the pelvis and vertebrae[4-6].

Recently, we have studied a patient who presented vertebral lesions and spinal compression secondary to B-cell NHL. As this type of lymphoma presentation has rarely been reported in the literature[5-9], we felt this merited publication.

Clinical Case Report

A 67-year-old male smoker with a history of carotid atheromatosis, who was admitted hospital after reporting weakness in the lower limbs. Two years before, he had suffered a lacunar cerebral infarction in a left radiated crown, which progressed without clinical sequalae. For the past year, he complained of mechanical-type pain in the lower back, radiating to his right side, which increased in intensity over the last week. This condition became resistant to analgesics and made it difficult for him to sleep. Three days before being admitted to hospital, he reported weakness and paresthesias in lower limbs, as well as difficulty initiating urination. For three months, he had also reported asthenia, anorexia, and unquantified weight loss. Upon examination, we found paraparesis of proximal predominance, with hyporeflexia and hypoesthesia, with sensorial level in T8. No adenopathies or visceromegalias were palpable, with the rest of the physical examination unremarkable. Among the analytical data was a slight leukocytosis (11,900/μL) with 77% segmented and 11% lymphocytes. Hemoglobin and platelets were normal, as was the routine biochemical study, including levels of calcium, albumin, phosphate and LDH. No abnormalities were observed in serum electrophoretic study and tumor markers (including β2-microglobulin) were normal. The study of the peripheral blood morphology was also normal.

A dorso-lumbar spine nuclear magnetic resonance (MRI) (Figure 1A) showed a loss of height of T8, which was occupied by a mass that extended to soft paravertebral tissues, especially towards the posterior vertebral and inward elements of the spinal canal, forming an epidural infiltration cuff that reached cranially until T7 and caudally until T10. All of this conditioned a segmental stenosis of the spinal canal, with deformity of the anterior contour of the medullary cord. Patches were also observed in T7 and T9, compatible with involvement of both vertebral bodies. Full-body computed tomography (CT) showed no mass or nodule suggesting malignancy or significant lymphadenopathy.

Positron emission tomography (PET/CT) showed the existence of an intense uptake suggestive of malignancy in the left T8 pedicle with destruction, as well as a slight hypermetabolism in vertebral bodies T7, T9 and T11 (Figure 1B). There were no other pathological outbreaks of uptake in other areas of the organism.

A CT-guided percutaneous biopsy of T8-dependent paravertebral mass was carried out. Its histological and immuno-histochemical study showed diffuse large B-cell lymphoma (Figure 2). No MYC, BCL2 and BCL6 rearrangements were detected. No infiltration or dysplasia data was observed in the bone marrow biopsy. The immunophenotypic study was normal.

Initially, the possibility of surgical decompression was considered, but this was ruled out after histological study. R-CHOP Chemotherapy and radiotherapy were commenced. However, the patient died after the first cycle due to septic shock attributed to *Staphylococcus Aureus*, secondary to infection of a grade III ulcer.

Discussion

Primary bone lymphoma (PBL), defined as the presence of one or more bone lesions with no evidence of nodal or ganglionic involvement, is uncommon. According to some studies, it represents only 0.9-5% of malignant bone tumors[1-3], less than 1% of lymphomas, and about 5% of lymphomas with extra-ganglionic involvement[4,5]. However, it should be taken into account that the definition of PBL is controversial. Most studies have included only patients with stage I and II, Ann Arbor staging system. In others, however, patients with stage IV disease (bone marrow involvement) have also been included. In addition, with improved staging procedures, especially with the combined use of computed tomography, MRI, and more recently, positron emission tomography (PET), the proportion of patients with systemic diagnosis (Stage IV, Ann Arbor staging) has increased. It usually occurs between the sixth and seventh decade of life and is more common in males than in females (3:2 ratio)[3]. It can manifest as a pathological fracture, especially in long bones, such as the femur. However, in cases where the spine is affected, the most frequent clinical presentation is lower back pain, which may be accompanied by neurological manifestations secondary to spinal compression, as occurred in our patient[5-9]. Unlike other types of lymphomas, PBL is not usually accompanied by affectation of the general condition, B-symptoms, or alterations in peripheral blood. In most cases, histopathological examination demonstrates the existence of diffuse B-lymphoma of large cells[3-5].

Bone lesions from lymphoma may take different patterns. The increase in density in a vertebral body ("ivory vertebra") is probably the most characteristic blast lesion, although it is also possible to see more or less diffuse osteo-sclerotic areas, which are sometimes associated with osteolytic phenomena[10]. However, destructive images that

Figure 1. MRI dorsum-lumbar column showing a T8-dependent right paravertebral mass extending from T7 to T10 (A). PET/CT scan showing the presence of a hyper-captive lesion in the left pedicle of T7, with slight hyper-metabolism in T8, T10 and T12 vertebral bodies and absence of pathological uptake to other levels (B)

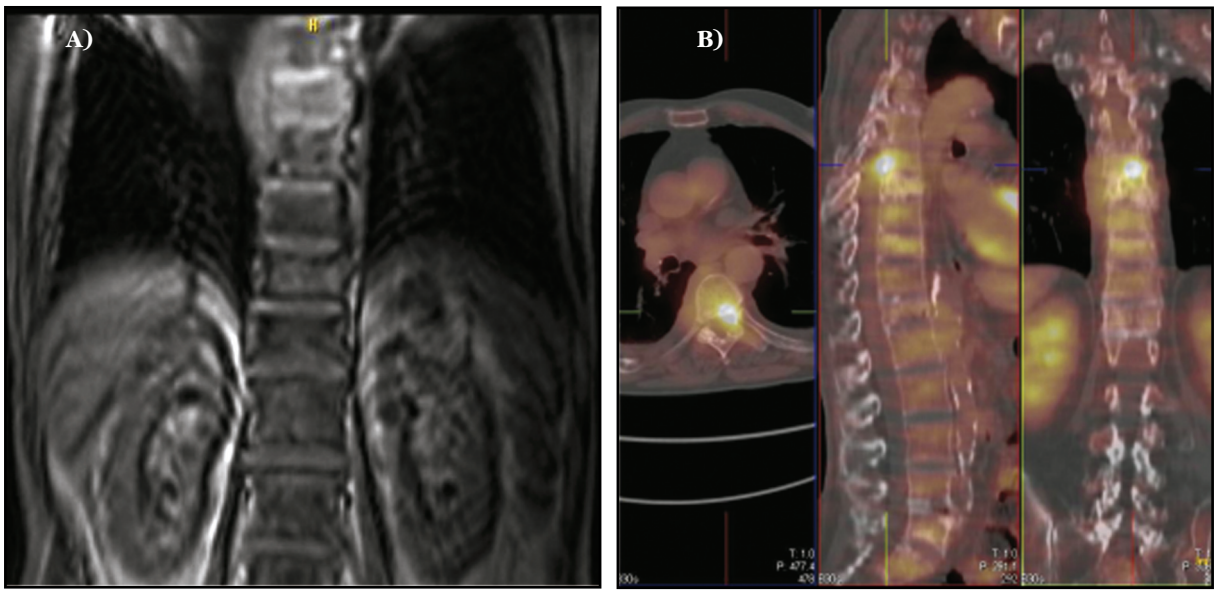
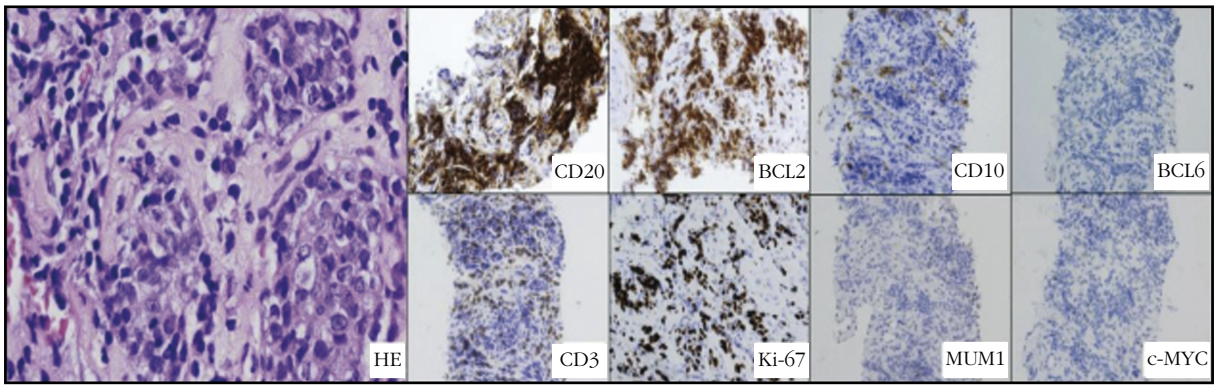


Figure 2. HE paravertebral mass (40x) in which a large-cell monomorphic, centroblastic-type lymphocyte with isolated eosinophilic nucleoli and nuclear indentations (B-lymphoma) is observed. Immuno-histochemical study (40x) in which CD20 and BCL2 were observed, with negativity for CD3, CD10, BCL6, MUM1 and c-MYC. The proliferative index (ki-67) was 60%



may be multifocal or isolated are more frequent. They predominate in the pelvis, the vertebrae and the long bones, arriving in these cases to break the cortex, infiltrating the adjacent soft tissues[11].

Treatment of PBLs is based on immuno-chemotherapy and radiotherapy, with surgery limited to obtaining samples for diagnosis and stabilization and fixation of possible fractures[12,13].

When there is vertebral and spinal involvement, as in our patient, surgical resection may be necessary, especially when the diagnosis is uncertain, but if preoperative biopsy confirms the presence of NHL, radiotherapy and/or chemotherapy alone can solve spinal compression and surgical intervention is not necessary[5,8,14-16].

The therapeutic response and prognosis are usually better in PBL than in lymphomas with secondary bone involvement, especially in the young, with little extensive lesions. This reinforces the importance of early diagnosis of these tumors[17,18].

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

Bibliography

1. The Non-Hodgkin´s Lymphoma Pathologic Classifications Project. National Cancer Institute sponsored study of classifications of non-Hodgkins lymphomas: summary and description of a working formulation for clinical usage. Cancer. 1982;49:2112-35.
2. Hogendoorn PCW, Kluin PM. Primary non-Hodgkin lymphoma of bone. In: Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F. (eds). WHO Classification of Tumours of Soft Tissue and Bone. 4th ed. Lyon: International Agency for Research on Cancer; 2013. p.316.
3. Jawad MU, Schneiderbauer MM, Min ES, Cheung MC, Koniaris LG, Scully SP. Primary lymphoma of bone in adult patients. Cancer. 2010;116:871-9.

4. Pettit Ck, Zukerberg LR, Gray MH, Ferry JA, Rosenberg AE, Harmon DC, et al. Primary Iymphoma of bone: a B-cell neoplasm with a high frequency of multilobulated cells. Am J Surg Pathol. 1990;14:329-34.
5. Maruyama D, Watanabe T, Beppu Y, Kobayashi Y, Kim SW, Tanimoto K, et al. Primary bone lymphoma: a new and detailed characterization of 28 patients in a single-institution study. Jpn J Clin Oncol. 2007;37:216-23.
6. Székely G, Miltényi Z, Mezey G, Simon Z, Gyarmati J, Gergely L Jr, et al. Epidural malignant lymphomas of the spine: collected experiences with epidural malignant lymphomas of the spinal canal and their treatment. Spinal Cord. 2008;46:278-81.
7. Becker S, Babisch J, Venbrocks R, Katenkamp D, Wurdinger S. Primary non-Hodgkin lymphoma of the spine. Arch Orthop Trauma. 1998;117:399-401.
8. Lyons MK, O'Neill BP, Marsh WR, Kurtin PJ. Primary spinal epidural non-Hodgkin's lymphoma: report of eight patients and review of the literature. Neurosurgery. 1992;30:675-80.
9. Çeçen DA, Tatarlı N, Turan Süslü H, Özdoğan S, Barışık NÖ. Primary Dural Spinal Lymphoma Presentation of a Rare Spinal Tumor Case. Case Rep Surg. 2015;2015:639253.
10. Fishman EK, Kuhlman JE, Jones RJ. CT of lymphoma: spectrum of disease. Radiographics. 1991;11:647-69.
11. Hernández JL, Olmos JM, Figols J, Riancho JA, González-Macías J. Lesiones osteolíticas femorales con tumoración de partes blandas e hipercalcemia como forma de presentación de un linfoma de estirpe B. An Med Intern. 2000;17:264-6.
12. Messina C, Christie D, Zucca E, Gospodarowicz M, Ferreri AJM. Primary and secondary bone Lymphomas. Cancer Treat Rev. 2015;41:235-46.
13. Ventre MB, Ferreri AJM, Gospodarowicz M, Govi S, Messina C, Porter D, et al. Clinical features, management, and prognosis of an international series of 161 patients with limited-stage diffuse large b-cell lymphoma of the bone (the IELSG-14 Study). Oncologist. 2014;19:291-8.
14. Stein ME, Kuten A, Gez E, Rosenblatt KE, Drumea K, Ben-Shachar M, et al. Primary lymphoma of bone -a retrospective study. Experience at the Northern Israel Oncology Center (1979-2000). Oncology. 2003;64:322-7.
15. Wong E, Portlock C, O'Brien J, DeAngelis L. Chemosensitive epidural spinal cord disease in non-Hodgkins lymphoma. Neurology. 1996;46:1543-7.
16. Baiocchi OC, Colleoni GW, Rodrigues CA, Barton D, Kerbauy FR, Garcia RJ, et al. Importance of combined-modality therapy for primary bone lymphoma. Leuk Lymphoma. 2003;44:1837-9.
17. Beal K, Allen L, Yahalom J. Primary bone lymphoma: treatment results and prognostic factors with long-term followup of 82 patients. Cancer. 2006;106:2652-6.
18. Wu H, Bui MM, Leston DG, Shao H, Sokol L, Sotomayor EM, et al. Clinical characteristics and prognostic factors of bone lymphomas: focus on the clinical significance of multifocal bone involvement by primary bone large B-cell lymphomas. BMC Cancer. 2014;14: 900.


se iomm
Sociedad Española de Investigación Ósea
y del Metabolismo Mineral


fe iomm
Fundación Española de Investigación Ósea
y del Metabolismo Mineral