Impact factor (JCR 2023): **0.8 / Q4**

October-December **2024** Volume **16** N.°-**4** Pages.: **113-153**

RMM

Revista de Osteoporosis y Metabolismo Mineral

Oficial Organ of Scientific Expression of the Sociedad Española de Investigación Ósea y del Metabolismo Mineral (SEIOMM) and of the Sociedad Iberoamericana de Osteología y Metabolismo Mineral (SIBOMM)

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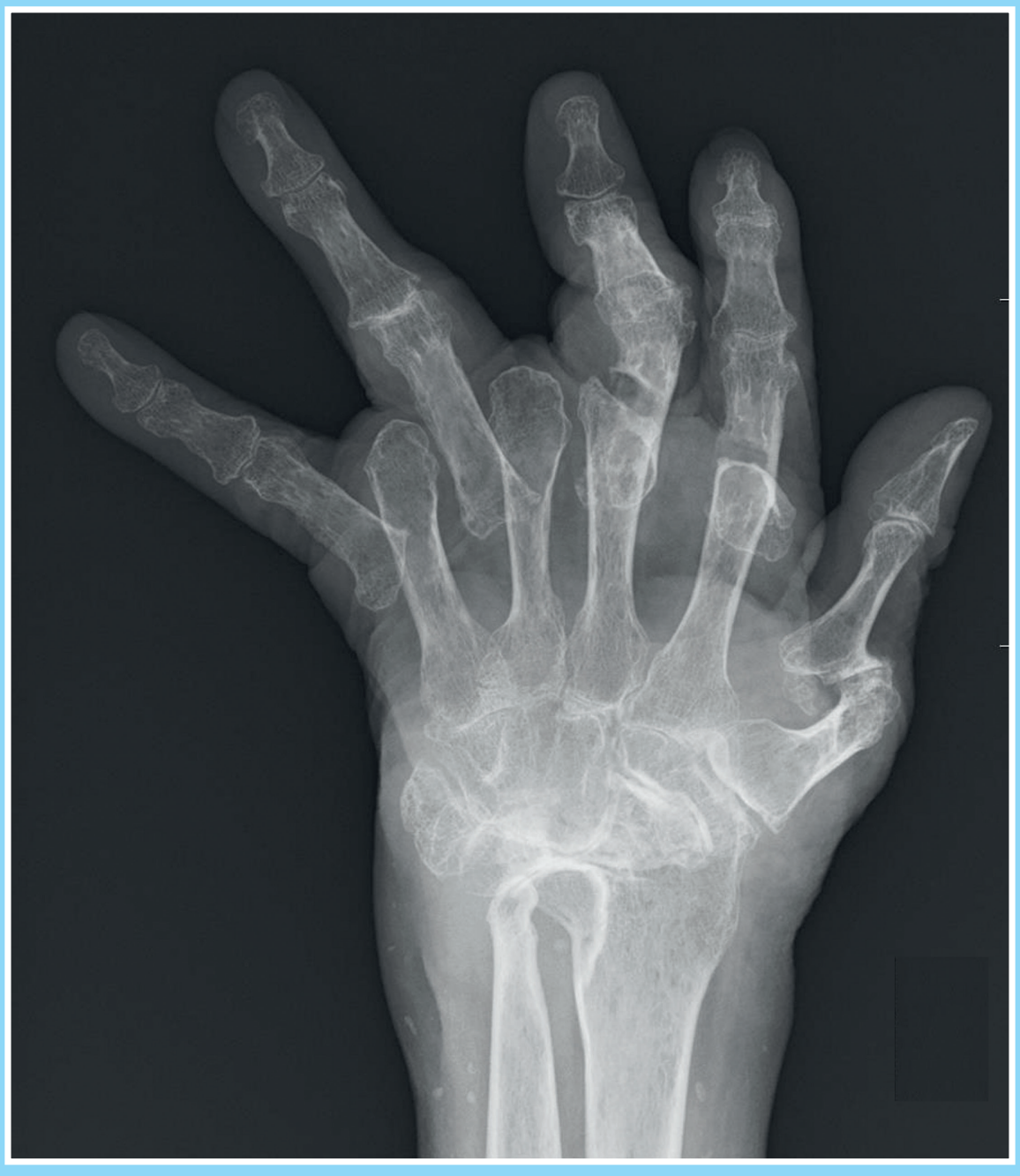

ARÁN Ediciones, S.L. ISSN (print version): 1889-836X. ISSN: (online version): 2173-2345

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Quarterly publication with 4 issues per year.

Indexes in which the journal is included:
Scielo, Web of Sciences, IBECS, Scopus, 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, ERIH plus, British Library, ROAD.
Impact factor (JCR 2023): 0,8 / Q4

ISSN (print version): 1889-836X. ISSN: (online version): 2173-2345
Legal Deposit: M-8158-2023

ARÁN EDICIONES, S.L.

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Summary

Vol. **16** ■ October-December ■ **N.º** 4

Originals

Special Article

Cover image:

Plain radiograph of the hand demonstrating skeletal changes associated with advanced rheumatoid arthritis: juxta-articular and diffuse osteoporosis, joint collapse, subchondral erosions, nd joint dislocations.

Hospital Universitario Marqués de Valdecilla. Santander, Spain

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Revista de Osteoporosis y Metabolismo Mineral

Original

The regulation of phosphate and its association with alterations in bone and mineral metabolism

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Abstract

Introduction and objective: although high levels of serum phosphate have been related to the risk of fracture and aortic calcification, it is not known if there is any association of urinary phosphate with the incidence of osteoporotic fracture and aortic calcification.

Material and methods: 141 postmenopausal women > 50 years of age underwent dorso-lumbar radiology that was repeated 4 years later, determining general biochemical markers and bone and mineral metabolism in blood and fresh urine, clinical and anthropometric parameters were collected. The appearance of new vertebral and non-vertebral fractures and new aortic calcification was radiographically confirmed. Women with estrogen and antiresorptive treatment > 3 months were excluded.

Results: 11 new non-vertebral fractures were detected (7 Colles, 2 hip and 2 in other locations) and 10 incident vertebral fractures confirmed radiographically. Body mass index, phosphaturia, creatinuria, phosphaturia/creatinuria and estimated glomerular filtration rate (eGFR) were significantly lower and age was higher in the fractured women. Increases of 10 mg/dL in phosphaturia were associated with 29 % fewer incident fractures [OR, 0.71; 95 % CI = (0.46-0.98)], after logistic regression adjusted for age, body mass index, creatinuria and eGFR. This effect was more marked with non-vertebral incident fractures [OR, 0.50; 95 % CI = (0.10-0.91)], while in vertebral this association was lost [OR, 0.83; 95 % CI = (0.54-1.14)]. Furthermore, 17 % of the cohort had new aortic calcifications. At a multivariate level, increases of 10 mg/dL of phosphaturia were associated with a lower incidence of aortic calcification [OR, 0.80; 95 % CI = (0.64-0.97)].

Conclusions: low phosphate levels seem to be associated with a higher incidence of osteoporotic fracture and aortic calcification in women. Phosphaturia could be an indicator of hormonal and renal effects on phosphate regulation and used as a risk factor for aortic fracture and calcification.

Keywords:
Phosphaturia.
Abdominal aortic calcification.
Osteporotic fracture.

Received: 05/07/2024 • Accepted: 21/10/2024

Funding: this original article was funded by a FEIOMM 2023 research grant, with Minerva Rodríguez García as the principal investigator.

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

Artificial Intelligence: the authors declare that they did not use artificial intelligence (AI) or any AI-based tools for the drafting of this article.

Rodríguez García M, Gómez Alonso C, Avello Llano N, García Gil-Albert C, Sobrino Díaz L, Naves Mendívil L, Montero Muñoz J, Palomo Antequera C, Naves Díaz M. The regulation of phosphate and its association with alterations in bone and mineral metabolism. Rev Osteoporos Metab Miner 2024;16(4):113-122

DOI: 10.20960/RevOsteoporosMetabMiner.00051

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INTRODUCTION

Osteoporotic fractures constitute a significant health and economic issue for public health systems (1) and are associated with high morbidity and mortality in the general population.

There is increasing evidence that elevated phosphate levels are linked to an increased risk of fracture (2,3). In fact, two large population cohort studies, the Rotterdam Study and the MrOS (Osteoporotic Fracture Study in Men), reported that increased serum phosphate levels were associated with fracture risk in participants without chronic kidney disease (3). Recently, this same effect has been observed in both male and female dialysis populations, suggesting that lowering serum phosphate levels may prevent fractures (4). In fact, an observational study of 13,427 dialysis patients showed that those not treated with phosphate binders had a 20 % higher risk of fracture compared to those who were treated (5). These findings are supported by evidence from animal models in which dietary phosphate increase induces bone fragility, even when circulating phosphate concentrations remain within the normal range (6).

The direct association between bone tissue deterioration and the development of arterial calcification has long been acknowledged (7,8). The role of phosphate in the development and progression of arterial calcification is well established in the presence of impaired renal function (9), but also in the general population (10).

As is well known, fragility fractures are associated with aging, but they occur more frequently in women. One of the main factors attributed to this gender difference is the estrogen deprivation that occurs in women after menopause, which leads to accelerated bone tissue loss, ultimately resulting in greater susceptibility to fragility fractures (11). Cardiovascular disease and its clinical manifestation with the onset of arterial calcification in women also begin to manifest with menopause and the loss of estrogen (12).

Postmenopausal women with estrogen depletion exhibit hyperphosphatemia due to increased proximal tubular phosphate reabsorption (13). However, no studies have ever analyzed whether there is any association between urinary phosphate levels and the presence of fractures and/or arterial calcifications. Therefore, the aim of this study was to evaluate the effect of urinary phosphate on the incidence of osteoporotic fractures and aortic calcification in women from the general population.

MATERIAL AND METHODS

We selected a random sample of 316 women over 50 years old from the municipal registry of Oviedo (Asturias, Spain). The protocol for this study included completing a questionnaire on risk factors related to osteoporosis, two lateral thoracolumbar X-rays, a densitometry study of the lumbar spine and hip, anthropometric measurements (height and weight) to calculate body mass index (BMI), a gynecological and drug history. All subjects had sufficient ambulatory capacity to climb two floors without an elevator, and 99 % lived in their own home.

After 4 years, the participants were invited to repeat the radiological study, densitometric measurements, anthropometric measurements, osteoporosis risk factor questionnaire, and a biochemical study. A total of 213 women participated in the second control, and 168 agreed to undergo the biochemical study. A total of 18 women were excluded from the analysis for having received osteoporotic or estrogenic treatment, and another 9 were excluded for still being in their menstrual periods. Data were available for 141 women at both the start and after 4 years.

INCIDENCE OF OSTEOPOROTIC FRACTURE

For the diagnosis of new vertebral fractures, the baseline radiographs were compared with the follow-up X-rays by two independent readers who performed a qualitative radiological evaluation without knowing the clinical conditions of the evaluated subjects. A new or incident fracture was defined as a reduction, visible to the naked eye, in any of the vertebral body heights vs the baseline X-ray, following the Genant method (14). If there was no agreement between the 2 readers, the fracture was defined by consensus after joint review. The interobserver reproducibility (Cohen's kappa coefficient) of the 2 readers in defining incident fractures was 0.82, indicating good reproducibility. All non-vertebral osteoporotic fractures, excluding those of the skull and extremities, were confirmed by X-ray.

EVALUATION OF INCIDENT VASCULAR CALCIFICATION

The presence of new aortic calcification was determined by comparing the baseline X-rays with those taken 4 years later.

Abdominal aortic calcification was evaluated by 2 independent investigators and categorized as grade 0 (absent), grade 1 (mild-moderate), and grade 2 (severe). Isolated punctate calcifications, a linear calcification visible in < 2 vertebral bodies, or a dense calcified plaque were defined as mild-moderate calcification (15). The presence of a linear calcification visible along at least two vertebral bodies and/or the presence of > 2 dense calcified plaques was defined as severe calcification. The intra- and interobserver

reproducibility (Cohen's kappa coefficient) for analyzing the radiographs to define incident aortic calcification was 0.78 and 0.73, respectively, indicating good reproducibility (15).

DENSITOMETRIC EVALUATION

Bone mineral density (BMD) was measured using a Hologic® QDR-1000 DXA densitometer (Hologic Inc., Waltham, MA, USA). In all cases, antero-posterior lumbar spine (L2-L4) and right femur BMD were analyzed. The coefficient of variation (CV) was 1.2 % and 1.9 %, respectively (15). Quality control and precision were performed daily with a lumbar spine phantom, which yielded a CV of 0.0 ± 0.1 %. In the fourth year, BMD was determined in the same areas used in the first study, and the percentage change between both measurements was used to assess changes in BMD.

BIOCHEMICAL ANALYSIS

No biochemical analysis was performed at baseline. After 4 years, blood and fresh urine samples were collected from each participant in a fasting state. Once the serum and urine were separated, they were stored at -80 °C until quantification. Creatinine serum levels, estimated glomerular filtration rate, calcium, phosphorus, albumin, total alkaline phosphatase, acid phosphatase, and tartrate-resistant acid phosphatase were measured. In urine, creatinine, phosphorus, and calcium levels were determined. Both serum and urine were analyzed using an autoanalyzer (Hitachi Mod. 717, Ratigen, Germany).

Serum levels of calcidiol (25OHD) were measured by prior extraction with acetonitrile (IDS, Ltd., Bolton, UK), with intra- and inter-assay CVs of 5.2 % and 8.2 %, respectively. Levels of 1,25-dihydroxyvitamin D were measured by radioimmunoassay (IDS, Ltd.); intra- and inter-assay CVs were 6.5 % and 9 %, respectively. Intact PTH levels were measured by radioimmunoassay (Nichols Institute, San Juan Capistrano, CA, USA), with intra- and inter-assay CVs of 2.6 % and 5.8 %, respectively.

All studies were conducted in accordance with the principles outlined in the Helsinki Declaration and were formally approved by the Clinical Trials Committee of the Principality of Asturias.

STATISTICAL ANALYSIS

Data analysis was performed using SPSS version 17.0 for Windows. Quantitative variables were analyzed using Student's t-test. Qualitative variables were analyzed using chi-square tests. Pearson correlations were performed between quantitative variables.

To analyze, at a multivariate level, the effect of phosphaturia on the incidence of vertebral and non-vertebral osteoporotic fractures, as well as on the incidence of aortic calcification, logistic regression was used, adjusted for variables that were statistically significant in the univariate model.

RESULTS

In the 4-year follow-up period, 11 new non-vertebral fractures were detected (7 Colles fractures, 2 hip fractures, and 2 in other locations), along with 10 incident vertebral fractures. All fractures were confirmed radiographically.

Table I shows the demographic, anthropometric, and biochemical variables at 4 years from the start of the study in women with incident vertebral and non-vertebral fractures. Women with incident fractures had significantly lower BMI, phosphaturia, creatinuria, and estimated glomerular filtration rate (eGFR), while their age was significantly higher.

Considering that women with incident fractures were associated with significantly lower phosphaturia levels at a univariate level, a logistic regression model was conducted using incident osteoporotic fracture as the dependent variable and phosphaturia as the independent variable, adjusted for age, BMI, creatinuria, and eGFR. Increases of 10 mg/dL in phosphaturia were associated with 29 % fewer incident fractures [odds ratio (OR, 0.71; 95 %CI, 0.46-0.98)].

Table II shows the demographic, anthropometric, and biochemical variables at 4 years from the start of the study, separated by women with and without incident non-vertebral fractures. Women with fractures had significantly lower BMI, phosphaturia, and creatinuria. The logistic regression analysis, adjusted for BMI and creatinuria, showed that increases of 10 mg/dL in phosphaturia were associated with 50 % fewer incident fractures [OR, 0.50; 95 %CI, 0.10-0.91)].

Table III shows the demographic, anthropometric, and biochemical variables at 4 years from the start of the study, separated by women with and without incident vertebral fractures. In women with fractures, significantly lower phosphaturia and creatinuria levels were observed. However, in the logistic regression analysis adjusted for creatinuria, phosphaturia (increases of 10 mg/dL) was not associated with the incidence of vertebral fractures [OR, 0.83; 95 %CI, 0.54-1.14)].

In light of the previous results and considering that phosphaturia had a greater effect on non-vertebral fractures than on vertebral fractures, the percentage changes in bone mineral density at the lumbar level and femoral neck were analyzed between the 2 cross-sectional time points.

Table I. Demographic, anthropometric, and serum and urinary markers of general and bone and mineral metabolism in women with and without incident vertebral and non-vertebral fractures

Variables	Fractured (*n* = 17)	Non-fractured (*n* = 124)	*p-value*
Age (years)	67.8 ± 8.1	63.6 ± 7.9	*0.044*
BMI (kg/m^2)	26.2 ± 2.4	29.0 ± 4.7	*0.001*
Creatinine (mg/dL)	0.95 ± 0.19	0.92 ± 0.11	0.481
Estimated glomerular filtration rate (eGFR) (mL/min)	52.9 ± 10.9	66.8 ± 16.9	*< 0.001*
Serum phosphorus (mg/dL)	3.51 ± 0.47	3.62 ± 0,.41	0.304
Serum calcium (mg/dL)	9.34 ± 0.16	9.40 ± 0.33	0.181
Calcidiol (ng/mL)	14.3 ± 7.2	14.9 ± 8.9	0.774
Calcitriol (pg/mL)	36.0 ± 10.1	38.1 ± 12.8	0.531
Total alkaline phosphatase (U/L)	181 ± 59	186 ± 88	0.821
PTH (pg/mL)	63.7 ± 37.4	54.8 ± 21.4	0.360
Acid phosphatase (U/L)	2.79 ± 0.65	2.83 ± 0.92	0.839
Tartrate-resistant acid phosphatase (TRAP) (U/L)	2.26 ± 0.62	2.16 ± 0.70	0.567
Serum albumin (g/L)	44.6 ± 2.3	45.3 ± 2.1	0.228
Urinary phosphorus (mg/dl)	47.8 ± 15.0	73.8 ± 33.8	*< 0.001*
Urinary calcium (mg/dl)	12.7 ± 6.4	14.1 ± 8.4	0.509
Urinary creatinine (mg/dL)	63.6 ± 26.8	79.9 ± 37.6	*0.034*
Tubular phosphate reabsorption (TPR)	0.78 ± 0.08	0.76 ± 0.07	0.258

Table II. Demographic, anthropometric, and serum and urinary markers of general and bone and mineral metabolism in women with and without incident non-vertebral fractures

Variables	Fractured (*n* = 9)	Non-fractured (*n* = 132)	*p-value*
Age (years)	67.3 ± 8.8	63.9 ± 7.9	*0.214*
BMI (kg/m^2)	26.7 ± 2.3	28.9 ± 4.6	*0.033*
Creatinine (mg/dL)	0.99 ± 0.22	0.92 ± 0.11	0.352
Estimated glomerular filtration rate (eGFR) (mL/min)	54.2 ± 12.3	66.0 ± 16.9	*0.054*
Serum phosphorus (mg/dL)	3.43 ± 0.42	3.62 ± 0.41	0.195
Serum calcium (mg/dL)	9.32 ± 0.17	9.40 ± 0.33	0.500
Calcidiol (ng/mL)	14.0 ± 7.6	14.9 ± 8.8	0.780
Calcitriol (pg/mL)	38.0 ± 10.1	37.8 ± 12.7	0.972
Total alkaline phosphatase (U/L)	166 ± 71	187 ± 85	0.465
PTH (pg/mL)	76.1 ± 48,.7	54.5 ± 21.1	0.212
Acid phosphatase (U/L)	2.80 ± 0.79	2.83 ± 0.90	0.923
Tartrate-resistant acid phosphatase (TRAP) (U/L)	2.26 ± 0.72	2.16 ± 0.69	0.698
Serum albumin (g/L)	44.46 ± 18	45.3 ± 2.1	0.253
Urinary phosphorus (mg/dL)	42.2 ± 12.2	72.6 ± 33.3	*< 0.001*
Urinary calcium (mg/dL)	11.7 ± 6.5	14.1 ± 8.3	0.396
Urinary creatinine (mg/dL)	58.2 ± 19.1	79.3 ± 37.3	*0.012*
Tubular phosphate reabsorption (TPR)	0.78 ± 0.07	0.76 ± 0.07	0.345

Table III. Demographic, anthropometric, and serum and urinary markers of general and bone and mineral metabolism in women with and without incident vertebral fractures

Variables	Fractured (*n* = 10)	Non-fractured (*n* = 131)	*p-value*
Age (years)	68.5 ± 7.2	63.8 ± 8.0	*0.072*
BMI (kg/m^2)	25.7 ± 2.5	28.9 ± 4.6	*0.053*
Creatinine (mg/dL)	0.90 ± 0.12	0.92 ± 0.12	0.586
Estimated glomerular filtration rate (eGFR) (mL/min)	53.0 ± 9.9	66.1 ± 17.0	*0.007*
Serum phosphorus (mg/dL)	3.63 ± 0.47	3.60 ± 0.41	0.829
Serum calcium (mg/dL)	9.34 ± 0.15	9.40 ± 0.33	0.615
Calcidiol (ng/mL)	13.7 ± 7.1	14.9 ± 8.8	0.675
Calcitriol (pg/mL)	32.9 ± 10.2	38.2 ± 12.7	0.198
Total alkaline phosphatase (U/L)	213 ± 47	184 ± 87	0.289
PTH (pg/mL)	57.9 ± 25.6	55.6 ± 23.9	0.771
Acid phosphatase (U/L)	2.87 ± 0.64	2.83 ± 0.91	0.879
Tartrate-resistant acid phosphatase (TRAP) (U/L)	2.35 ± 0.68	2.16 ± 0.69	0.398
Serum albumin (g/L)	44.7 ± 2.7	45.3 ± 2.1	0.407
Urinary phosphorus (mg/dL)	50.1 ± 16.5	71.8 ± 33.5	*0.002*
Urinary calcium (mg/dL)	12.7 ± 6.2	14.0 ± 8.3	0.649
Urinary creatinine (mg/dL)	65.7 ± 31.0	78.6 ± 37.1	*0.286*
Tubular phosphate reabsorption (TPR)	0.79 ± 0.08	0.76 ± 0.07	0.188

None of the three bone segments analyzed correlated with phosphaturia: lumbar spine: r = -0.077, p = 0.431; femoral neck: r = -0.66, p = 0.482; total hip: r = 0.028, p = 0.764.

However, when associations between urinary phosphate and bone mineral density in the three segments were analyzed, only in the second cross-sectional analysis was no correlation found between lumbar BMD and urinary phosphate (r = 0.118, p = 0.229), but a correlation was found between femoral neck BMD and urinary phosphate (r = 0.239, p = 0.004), and between total hip BMD and urinary phosphate (r = 0.232, p = 0.006) (Fig. 1).

A total of 23 women (17.1 %) developed new aortic calcifications during the 4-year follow-up period. Table IV presents the demographic, anthropometric, and biochemical variables at 4 years from the start of the study, separated by women with and without incident aortic calcification, considering the presence of new calcification not seen on the baseline X-rays. Women with incident aortic calcification had significantly lower serum albumin and phosphaturia levels. Logistic regression analysis, adjusted for serum albumin, showed that increases of 10 mg/dL in phosphaturia were associated with a lower incidence of aortic calcification [OR, 0.80; 95 %CI, 0.64-0.97)]. It was also notable that increases of 1 mg/dL in serum albumin reduced the incidence of aortic calcification by 31 % [OR, 0.69; 95 %CI, 0.54-0.88)].

DISCUSSION

The results of this study present, for the first time in the literature, an association between decreases in phosphaturia and an increase in the incidence of osteoporotic fractures and aortic calcification. In fact, increases of 10 mg/dL in phosphaturia in postmenopausal women decreased the incidence of both vertebral and non-vertebral fractures by 29 %, with a much stronger effect observed in non-vertebral fractures, which occur in bones with a more cortical content, as shown by the significant correlation between phosphaturia and BMD in the femoral neck and total hip. This effect was not observed in lumbar BMD, which has a more trabecular content. On the other hand, increases of

Table IV. Demographic, anthropometric variables, and general serum and urinary markers of bone and mineral metabolism in women with and without incident aortic calcifications

Variables	Calcification (*n* = 23)	No Calcification (*n* = 110)	*p-value*
Age (years)	69.6 ± 6.1	68.0 ± 8.3	*0.296*
BMI (kg/m^2)	29.7 ± 4.4	28.6 ± 4.6	*0.314*
Creatinine (mg/dL)	0.90 ± 0.11	0.93 ± 0.13	0.296
Estimated glomerular filtration rate (eGFR) (mL/min)	67.1 ± 14.6	65.0 ± 17.4	*0.596*
Serum phosphorus (mg/dL)	3.64 ± 0.48	3.61 ± 0.39	0.775
Serum calcium (mg/dL)	9.34 ± 0.24	9.40 ± 0.33	0.390
Calcidiol (ng/mL)	14.6 ± 7.2	14.7 ± 8.6	0.976
Calcitriol (pg/mL)	40.3 ± 14.2	37.0 ± 11.3	0.223
Total alkaline phosphatase (U/L)	214 ± 169	180 ± 55	0.347
PTH (pg/mL)	60.7 ± 27.0	55.1 ± 33.8	0.320
Acid phosphatase (U/L)	2.70 ± 0.89	2.87 ± 0.90	0.436
Tartrate-resistant acid phosphatase (TRAP) (U/L)	2.08 ± 0.82	2.19 ± 0.67	0.495
Serum albumin (g/L)	44.1 ± 2.3	45.5 ± 2.1	0.004
Urinary phosphorus (mg/dL)	59.1 ± 26.9	73.8 ± 34.5	*0.047*
Urinary calcium (mg/dL)	13.0 ± 8.7	14.0 ± 8.2	0.591
Urinary creatinine (mg/dL)	69.6 ± 35.0	80.2 ± 37.8	*0.219*
Tubular phosphate reabsorption (TPR)	0.78 ± 0.06	0.75 ± 0.07	0.148

10 mg/dL in phosphaturia in this same cohort reduced the incidence of aortic calcification by 20 %.

At the biochemical level, PTH was not able to explain this effect. Serum PTH levels were found to be slightly, but not significantly, higher in women with either incident fracture or incident aortic calcification. This slight increase should have contributed to an increase in urinary phosphate excretion, something that not only did not happen, but even went in the opposite direction.

There are several mechanisms that could explain how phosphate might affect bone quality and strength. Regarding bone formation, inorganic phosphate can stimulate several regulatory molecules (phos antigen 1, osteopontin, insulin-like growth factor I, and sclerostin), which would inhibit Wnt/beta-catenin and osteoblast proliferation (16,17). Inorganic phosphate also affects bone resorption by limiting the survival and differentiation of osteoclasts, inducing changes in the expression of RANKL, miR-223, and osteoprotegerin (18-22).

However, in our study, we did not observe higher serum phosphate levels in the group of women with fractures, with urinary phosphate levels being the factor that marked the observed differences. The fact that we did not see any effect of serum phosphate on fracture incidence in women has been previously described in other epidemiological studies. In an elderly population study, the upper decile (D10) of serum phosphate in men had a 78 % higher risk of incident fracture (HR, 1.78; 95 %CI, 1.25-2.54), a relationship not found in women (HR, 1.09; 95 %CI, 0.83-1.44) (2). These results are similar to those of other studies where the association between high serum phosphate levels and incident fractures was substantially stronger in men than in women (3). The disparity between men and women could reflect a difference in sensitivity to high serum phosphate levels, keeping in mind that, in general, women have higher serum phosphate levels than men of similar age.

Unlike what has been described by other authors (23-25), we did not find any associations between serum phosphate levels and the incidence of aortic calcification.

Since the main biochemical regulators of phosphorus metabolism do not allow us to discern why we found this association between urinary phosphate and the

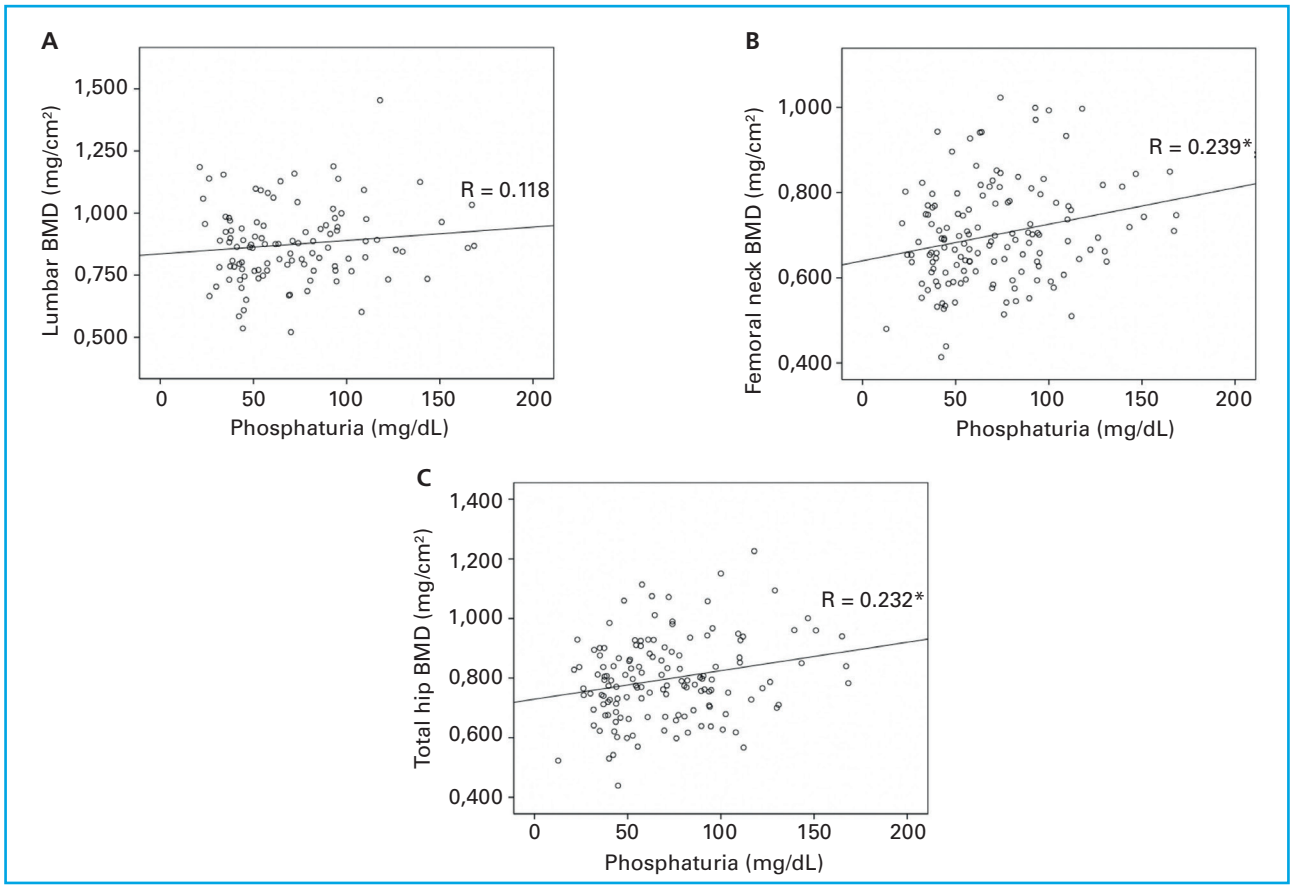

Figure 1. Correlations between phosphaturia and bone mineral density (BMD) in the second cross-sectional cut in lumbar spine (A); femoral neck (B); total hip (C). *$p < 0.05$.

incidence of fractures and/or aortic calcification, we need to consider other regulatory mechanisms. In this cohort of postmenopausal women, there is an estrogen deficit. It is known that estrogens are an important physiological regulator involved in the functional modulation of several hormones, suggesting that the mechanisms by which estrogens regulate mineral metabolism could be complex and involve both direct and indirect effects.

We should highlight the hypophosphatemic and hyperphosphaturic effects of estrogens in different clinical studies. Several studies have observed that estrogen administration in postmenopausal women is associated with hypophosphatemia, which in some cases is due to reduced phosphate reabsorption in the proximal tubule (26-28) and the negative regulation of NaPi-IIa in the proximal tubule (29). In contrast, decreases in estrogen levels are associated with an increase in serum phosphate (hyperphosphatemia) and an increase in tubular phosphate reabsorption in the proximal tubule with a decrease in urinary phosphate excretion (hypophosphaturia). Therefore, in the context of former studies, these data suggest that estrogens may directly or indirectly induce phosphaturia in humans (30).

There is strong evidence indicating that estrogens can exert a rapid, non-genomic effect in certain target tissues (31,32). It remains to be determined whether these pathways contribute to the phosphaturic effect of estrogens (33).

After menopause, the main estrogen of reproductive years, 17β-estradiol, decreases significantly, and estrone becomes the primary estrogen in tissues and circulation. Estrone is produced through the conversion of adrenal androstenedione by aromatase, primarily in adipose tissue. Estrone increases almost 2 times in obesity due to its greater aromatization in adipose tissue (34).

Serum levels of 17β-estradiol and estrone are more than double in obese individuals vs lean postmenopausal women (35). Therefore, we could hypothesize the existence of fat-induced estrogen production, which could contribute to women with overweight having extra estrogen secretion increasing phosphaturia compared to what happens in the typical osteoporotic woman who is thin and fragile. In fact, when analyzing the incidence of vertebral and non-vertebral fractures in the univariate analysis, BMI was significantly lower in those women with fractures.

The effect of phosphaturia on the incidence of aortic calcification, similar to that observed for incident fractures, is both interesting and surprising. However, we cannot ignore that both alterations are associated with age, which may be related to the possibility that in response to calcifying stimuli, vascular smooth muscle cells dedifferentiate into osteoblast-like cells capable of synthesizing osteogenic markers (36). It is also worth noting how decreases in serum albumin contribute to the increase in calcification, a phenomenon described by other authors (37).

We cannot rule out the existence of limitations in this study. First, we do not have serum estrogen levels that could explain these differences in phosphaturia. Second, we do not have serum FGF23 levels, which could provide additional value, but when the study was conducted, we did not have this serum marker and unfortunately, there is no sample available for testing. Third, phosphaturia was only measured in the second cross-sectional cut, limiting the associations found. Finally, we must not forget that the sample size is somewhat limited.

Nevertheless, despite acknowledging the limitations, this study also has strengths, such as being a prospective study with a follow-up participation rate above 50 %, at a time when epidemiological studies were not very common in our country. The prospective, rather than cross-sectional, nature of the study strengthens the validity of the results and their greater degree of association.

We can conclude that low phosphaturia seems to be associated with a higher incidence of osteoporotic fractures and aortic calcification in postmenopausal women. Regarding osteoporotic fractures, the effect seems more marked in bones with higher cortical than trabecular content. Whether serum estrogen levels contribute to this effect is something that should be confirmed in future studies. Based on these results, in elderly women, phosphaturia could be an indicator of hormonal and renal effects on phosphate regulation and could be used as another risk factor for osteoporotic fractures and aortic calcification.

ACKNOWLEDGMENTS

This work was partially funded by the European Vertebral Osteoporosis Study (EVOS), European Union (1991–1993); European Prospective Osteoporosis Study (EPOS), European Union (BIOMED 93-95), BMHI-CT 092-0182 (1993-1997); Health Research Fund (FIS 94/1901-E); ISCIII REDinREN network (RD06/0016/1013, RD12/0021/0023, RD16/0009/0017, RICORS2040 (RD21/0005/0019), National R&D Plan 2008-2011, State R&D Plan 2013-2016, Funded by the European Union – Next Generation EU, Recovery and Resilience Mechanism (MRR); European Regional Development Fund (ERDF), Science, Technology, and Innovation Plan 2013-2017 and 2018-2022 of the Principality of Asturias (GRUPIN14-028, IDI-2018-000152, IDI-2021-000080; IDE/2024/000706); Fundación Renal Íñigo Álvarez de Toledo (FRIAT).

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Revista de Osteoporosis y Metabolismo Mineral

Original

The activation of the purinergic system modulates the formation of foreign body giant cells in the presence of different metal alloys for clinical use

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Abstract

Objective: two major complications after arthroplasty are prosthetic joint infection, mainly due to *Staphylococcus aureus,* and the experience of a foreign body reaction by macrophages and foreign body giant cells (FBGCs) regardless of the infection. Our aim is to study the role of purinergic receptors with fusogenic function (P2X7, adenosine A1 and A2A receptors) in the formation of *S. aureus*-induced FBGCs and their possible differential modulation in the presence of Ti–6Al–4V and Cr-Co-Mo alloys.

Methods: RAW264.7 cells were differentiated to FBGCs with 20 ng/mL of IL-4 in the presence of adhered unviable *S. aureus,* metal alloys and/or CGS21680/ZM241385 1 µM. Cell supernatant was collected for nucleotide analysis by HPLC as well as cytokine expression, and cells were lysed for RNA expression.

Results: the presence of *S. aureus* induces an increase in FBGCs formation in a concentration-dependent manner. Furthermore, phalloidin staining demonstrated that both Ti–6Al–4V and Cr-Co-Mo alloys reduce the formation of FBGCs. The expression of adenosine A1 and A2A receptors increased after 5 days of differentiation in the presence of *S. aureus,* and this expression was enhanced with metal alloys. HPLC analyses showed an increase in adenosine in the presence of Ti–6Al–4V and Cr-Co-Mo alloys while ATP was not changed in any of the conditions. The presence of metal alloys induced an increased in IL1β, IL-6 and RANTES.

Conclusions: the increased levels of adenosine and the adenosine A2A receptor induced by the presence of Ti–6Al–4V and Co-Cr-Mo alloys would be responsible for the inhibition of cell fusion and the following reduction of *S. aureus*-induced FBGCs.

Keywords: Adenosine. A2A receptor. FBGCs. Metal alloys. Prosthetic joint infection.

Received: 08/08/2024 • Accepted: 14/11/2024

Authors´ contributions: AM designed the experiments, performed experiments, analyzed and interpreted the results, drafted and revised the manuscript. JJA-C and ET-S were responsible of bacterial preparation and plate coating, interpreted the results, drafted and revised the manuscript. MM-B. performed HPLC analyses, interpreted the results, drafted and revised the manuscript. AC, MA, JJD supplied and prepared the samples of both alloys for the present study, edited and revised the manuscript, RL, GH-B and JE interpreted the results, edited and revised the manuscript.

Data availability statement: the original contributions presented in the study are included in the article/supplementary data; further inquiries can be directed to the corresponding author.

Conflict of interest: J. E. received travel grants from Pfizer and conference fees from bioMérieux and Heraeus. The funders had no role in the design of the study, the collection, analyses, or interpretation of data; the writing of the manuscript, or in the decision to publish the results. The remaining authors declare no conflict of interest.

Artificial intelligence: the authors declare that they did not use artificial intelligence (AI) or any AI-based tools for the drafting of this article.

Aguilera-Correa JJ, Marco-Bonilla M, Torrecilla-Sábada E, Conde A, Arenas MÁ, de Damborenea JJ, Largo R, Herrero-Beaumont G, Esteban J, Mediero A. The activation of the purinergic system modulates the formation of foreign body giant cells in the presence of different metal alloys for clinical use. Rev Osteoporos Metab Miner 2024;16(4):123-132

DOI: 10.20960/RevOsteoporosMetabMiner.00058

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INTRODUCTION

Arthroplasty has improved the quality of life of millions of patients and is highly effective, although implant-related complications can arise (1). Prosthetic joint infection (PJI) is one of the most important complications with a high morbidity and mortality rates, and associated costs. The incidence of PJI varies across countries from 0.5 % up to 2 % (2). Staphylococci are the most common etiological agents associated with PJI, with *Staphylococcus aureus* (30-40 %) and *S. epidermidis* being 2 of the main microorganisms involved (3). PJI results from the ability of microorganisms to develop a biofilm, a conglomerate of bacterial cells of, at least, 1 species that adheres to a surface or interface with complex socio-microbiological interactions among them (4).

Immediately after implantation with and without a perioperative PJI, the implantation surface is coated with plasma proteins that direct cellular adhesion and activation. Last step in the prosthesis implantation process is a foreign body reaction, that occurs when the inflammatory and wound healing responses have occurred including macrophages and foreign body giant cells (FBGCs) (9). These FBGCs are present on the surface of the implants throughout the life of the implant and produce stress cracks and oxidative damage (7). These macrophages and FBGCs secrete cytokines that in the early stages will modulate neutrophils and lymphocytes recruitment and activation (8). IL-4 and IL-13, both secreted by CD4+ T cells, are key cytokines for the formation of FGBCs involved in material degradation and implant rejection (9). In each one of the 4 phases for cell fusion, similar and different mechanisms for the formation of FBGCs or osteoclasts (multinucleated bone cells with the function of resorbing bone) have been described (10). At the end of this process, adherent macrophages and FBGCs induce biomaterial degradation with ensuing clinical device failure (5). Therefore, it is still being studied whether different materials respond differently to FBGCs adherence and how this biomaterial surface can be modulated to alter the presence and activity of adherent macrophages and FBGCs (11).

Purinergic signalling is also activated in cell fusion, especially in inflammation. Pharmacologically, 2 families of purinergic receptors are known: P1 receptors selective for adenosine, and P2 receptors selective for purine and pyrimidine nucleotides and dinucleotides (11,12). Adenosine is generated extra- and intra-cellularly by the hydrolysis of adenine nucleotides and performs its physiological functions through the activation of G-protein coupled transmembrane receptors (A1, A2A, A2B and A3), whose tissue distribution, pharmacological profile and effects are different (12). Purine and pyrimidine nucleotides activate 2 types of P2 receptors: P2X receptors—ionotropic, ion-linked channels—and P2Y receptors—metabotropic, G protein-coupled (11). The role of several P1 and P2 receptors in bone cells is well-known. The adenosine A1 receptor is critical and essential for differentiation and function of osteoclasts (13), while the adenosine A2A receptor induces an inhibition of osteoclast formation and function both *in vitro* (18) and in the osteolysis model (16). The adenosine A2B receptor is involved in osteoblast differentiation and function, with a more ambiguous role for the A2A receptor (16,17). Activation of adenosine A2A receptor both directly (by specific agonist) or indirectly (by drugs that increase extracellular adenosine levels by blocking its transport) promotes bone formation at levels similar to BMP-2 (bone morphogenic protein 2) (17). Among P2 receptors, P2X7 is especially relevant as its expression is positively regulated by pro-inflammatory cytokines (18). The P2X7 receptor is activated and is involved in cell fusion, where it intervenes in the late phases of membrane fusion and establishes union bridges between the cytoplasm of adjacent cells (19).

In orthopaedic surgery and traumatology, the most widely used medical alloys are Ti–6Al–4V and Co-Cr-Mo. Ti–6Al–4V alloy has favorable biocompatibility, mechanical properties and corrosion resistance (20). Furthermore, Co-Cr-Mo alloy exhibits high biocompatibility levels, with exceptional properties including abrasion, cracking-corrosion, pitting and wear resistance, malleability, and a high fatigue resistance and ductility (21).

Surprisingly, we know nothing of the effect that the presence of bacteria, such as *S. aureus* may or may not have during the formation process of these FBGCs. Therefore, we aim to investigate if variations in the metal alloys of the implants would change the recruitment of macrophages to the implantation area and, therefore, the formation of FBGCs since they would modulate the cell fusion process. More specifically, we believe that a differential activation of the purinergic system may be involved, and especially the P2X7 receptor and adenosine A1 and A2A receptors, as they are involved in the fusion of macrophages to osteoclasts.

METHODS

MATERIALS

An 18-mm diameter bar of ELI grade Ti–6Al–4V manufactured to ASTM F136-02, was supplied by Surgical Co., SAU (Valencia, Spain). The rod was cut into 2 mm thick disc specimens and ground through successive grades of SiC paper up to 2000 grade. To achieve a smooth surface with a final controlled roughness, chemical-mechanical polishing was then carried out in a commercial colloidal silica suspension (OP-S Suspension. 0.25 μm from Struers, Copenhagen, Denmark) with hydrogen peroxide with a volume ratio of 9:1.

A rod of Co-Cr-Mo alloy with a diameter of 19 mm was supplied by Carpenter Technology Corp. (Philadelphia, PA, USA), which was melted and tested in accordance with ASTM F1537-11. The rod was cut into 2 mm thick discs. One side of each disc was ground on silicon carbide paper and polished to a 3 μm diamond finish. The chemical composition of both alloys has been described elsewhere (22).

CELL LINES

Immortal murine macrophage line RAW264.7 (ATCC, LGC Standards S.L.U., Barcelona, Spain) were used. Cell culture was maintained in alpha MEM with 10 % FBS and 1 % penicillin/streptomycin and maintained in a humidified chamber at 37 °C, in 95 % air and 5 % CO_2.

The *S. aureus* strain chosen was a small colony variant strain (Sa35) isolated from a total hip PJI of a 79 years-old woman identified in the Clinical Microbiology Department at the Hospital Universitario Fundación Jiménez Díaz (Madrid, Spain). Several colonies of Sa35 grown on chocolate-blood agar were inoculated in an 8-mL tube of brain-heart infusion (BHI, BD, New Jersey, United States) at 37 °C for 24 h, then centrifuged at 3500 rpm at 22 °C for 10 min. The supernatant was removed, and the pellet was washed 3 times with sterile 0.9 % NaCl saline solution (SS) (B. Braun, Melsungen, Germany) and then resuspended and diluted in SS to get a bacterial solution (approximately, 1.61 ± 0.22·10^9 CFU/mL). The bacterial solution was autoclaved at 121 °C for 20 min. Both types of alloy coupons were incubated in 5 mL of this 1:100-bacterial solution in a sterile nontreated six-well plate (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 24 h to allow bacterial adhesion. Afterwards, the supernatant was discarded, and the metal samples were used in the next described experiment.

FOREIGN BODY GIANT CELLS (FBGCs) FORMATION

To determine the optimal conditions for infection by *S. aureus* in the RAW264.7 cells, proliferation (alamar blue, Bio-Rad, Madrid, Spain) was performed on the cells at 72 hours, in 3 different culture medias: *a)* alpha MEM with 10 % FBS without penicillin/streptomicin; *b)* RPMI with 5 % FBS without penicillin/streptomycin; and *c)* RPMI with 50 % bovine serum albumin (BSA) (*n* = 5).

A total of 10,000 RAW264.7 cells were differentiated with 20 ng/mL of IL-4 in the presence of adhered unviable*S. aureus* (1.61 ± 0.22·107, 106 and 105, 10^6 and 10^5 CFU/mL) for 5 days for giant cell formation. In some of the wells the co-culture will be performed in the presence of the material of the 2 above-mentioned alloys: Ti–6Al–4V and Co-Cr-Mo, and other well will be incubated with selective adenosine A2A agonist (CGS21680) and antagonist (ZM241385) 1 μM each. Cells were stained using the quick panoptic stain (PanReac Applichem, ITW Reagents, Barcelona, Spain) a non-vital, differential stain based on the May Grünwald-Giemsa staining. To check FBGCs formation in the metal alloys, phalloidin staining was performed when RAW264.7 cells were differentiated with 20 ng/mL of IL-4 in the presence of adhered unviable *S. aureus* (1.61 ± 0.22·10^7 CFU/mL) for 5 days. Briefly, cells were fixed with 4 % PFA, blocked in PBS BSA 1 % and 0.1 % Triton X-100 for 30 minutes and stained Alexa Fluor 555-phalloidin (Invitrogen, Fisher Scientific, Madrid, Spain) for 30 minutes.

NUCLEOTIDE CONCENTRATION DETERMINATION VIA HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

A total of 10,000 RAW264.7 cells were differentiated with 20 ng/mL of IL-4 in the presence of adhered unviable *S. aureus* (1.61 ± 0.22·10^7 CFU/mL) alone or in the presence of Ti–6Al–4V and Co-Cr-Mo for 3 or 5 days and supernatant were collected (*n* = 8 each). Samples were treated with EHNA and dipyridamole 1 μM each to avoid adenosine degradation/cellular uptaken. Protein denaturation and HPLC analysis were performed as described previously by Vivero-Lopez et al. (23). Briefly, a heat shock step was performed for 2 min at 98 °C, and samples were centrifuged at 13 000 g for 10 min at 4 °C. Supernatants were collected and stored at -80 °C until use. Inosine, adenosine, AMP, ADP and ATP concentrations were determined via high-performance liquid chromatography (HPLC) using a liquid chromatography with a reversed phase column (Agilent 1100 Series Liquid Chromatography) and a UV detector set at 254 nm. The buffer (0.1 mol/l KH_2PO_4 pH 7.5, 18 % acetonitrile) was run at 1.5 mL/min for 20 min. Compounds were identified and quantified by their retention times and peak areas of known standards, calibrated via spectrophotometry. The results are expressed as the mean ± standard error of the mean (SEM). All results were corrected according to heat shock lost and calculated as a percentage of basal cells.

CYTOKINE AND CHEMOKINE ARRAY

A total of 10,000 RAW264.7 cells were differentiated with 20 ng/mL of IL-4 in the presence of adhered unviable *S. aureus* (1.61 ± 0.22·10^7 CFU/mL) alone or in the presence of Ti–6Al–4V and Co-Cr-Mo alloys for 5 days and supernatant were collected (*n* = 5-6 each). Cytokines were measured using the Mouse Cytokine Array Q1 (QAM-CYT-1-4, RayBiotech, CliniSciences, Madrid, Spain) which, simultaneously, detects 20 cytokines. The cytokines concentration was measured by fluorescence using GenePix 4000B following the manufacturer´s recommendations.

RNA ISOLATION AND RT-PCR

Total RNA was isolated from cultures using TRIzol reagent based on the manufacturer's protocol. RNA (1 µg) was reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (2.5 U/µL), with RNase Inhibitor 1 U/µL, Random Hexamers 2.5 U/µL, $MgCl2^5$ mM, PCR buffer II 1× and dNTPs 1 mM (Applied Biosystems, Foster City, CA, USA). Relative analysis of gene expression was performed via real-time RT-PCR on a Step One Plus with Power UP SYBR Green MasterMix (Applied Biosystems). The primers used are listed in table I. Gene expression levels were calculated with the ΔΔCt method.

Table I. List of primers used for RT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
A2AR	TCGCCATCCGAATTCCACTC	TTTGTGCCCACAGATCTAGCC
A2BR	ATGGGCCAGATTAGGAGCAC	CTCCAAAAGGGGACCCAGTC
P2X7	GACAAACAAAGTCACCCGGAT	CGCTCACCAAAGCAAAGCTAAT
GAPDH	CTACACTGAGGACCAGGTTGTCT	GGTCTGGGATGGAAATTGTG

STATISTICAL ANALYSES

Statistical significance among groups was determined using one-way ANOVA and Bonferroni post-hoc test. All statistics were calculated using GraphPad® software (GraphPad, San Diego, CA, USA).

RESULTS

THE PRESENCE OF ADHERED UNVIABLE *S. AUREUS* INDUCES AN INCREASE IN THE FORMATION OF FBGCs THAT IS ABROGATED IN THE PRESENCE OF METAL ALLOYS

To understand the role of *S. aureus* in the formation of FGBCs, we first tested the most suitable media for the growth of RAW264.7 cells and *S. aureus*. Figure 1A illustrates how RAW264.7 cells proliferated adequately when they were seeded in αMEM medium with 10 % FBS and without antibiotics, while RPMI media with 5 % FBS but without antibiotics and RPMI with 50 % BSA, were not adequate for RAW264.7 cells proliferation. Afterwards, we tested the number of RAW264.7 cells and the concentration and IL-4 necessary for the correct differentiation of FBGCs. Figure 1B illustrates that the best formation of FBGCs was achieved with 10,000 RAW264.7 cells and 20 ng/mL of IL-4. Finally, we tested whether the presence of *S. aureus* modified FBGCs formation. We observed that the presence of *S. aureus* induced an increase in the formation of FBGCs in a concentration-dependent manner (54 ± 6 % increase for $1.61 \pm 0.22 \cdot 10^7$ CFU/mL, $p < 0.0001$).

Therefore, we decided to conduct all experiments in αMEM with 10 % FBS and without antibiotics, 10,000 RAW264.7 cells, 20 ng/mL of IL-4 and $1.61 \pm 0.22 \cdot 10^7$ CFU/mL.

When RAW264.7 cells and *S. aureus* where co-cultured in the presence of IL-4 and the metal alloys, immunofluorescence staining with phalloidin demonstrated that both Ti–6Al–4V and Co-Cr-Mo alloys reduced the formation of FBGCs in the presence of $1.61 \pm 0.22 \cdot 10^7$ CFU/mL *S. aureus* (Fig. 1D).

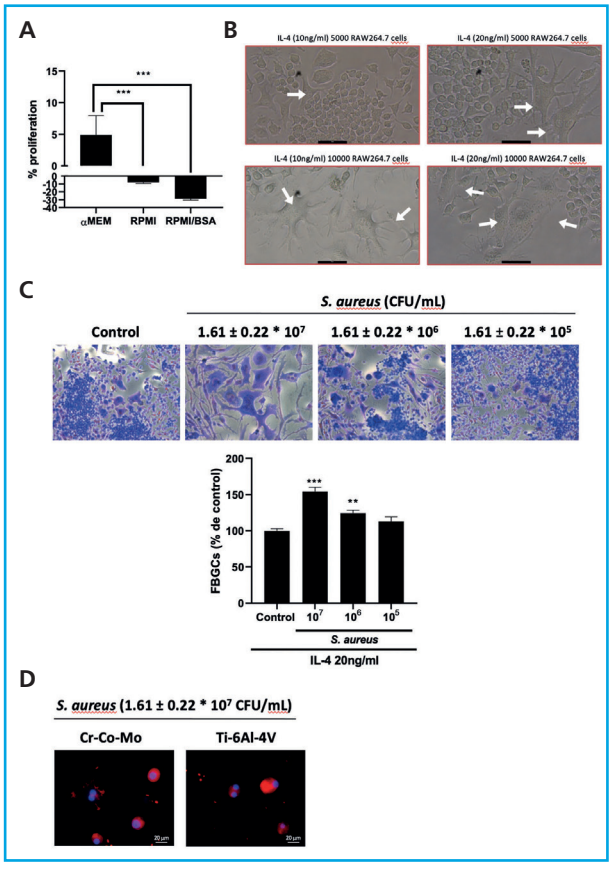

Figura 1. Adherent unviable *S. aureus* induces formation of FBGCs and presence of metal alloys inhibiting it. A. Optimal culture media condition determination. B. Determination of Raw264.7 cell number and IL-4 concentration optimal for culture. White arrows indicate the presence of giant cells. C. Panoptic staining representative images and quantification for FBGCs formation with adherent unviable *S. aureus.* D. Representative images for FBGCs formation with adherent unviable *S. aureus* in the presence of IL-4 and Ti–6Al–4V and Co-Cr-Mo alloys. All images were taken at 40x magnification. Data are presented as the mean ± SEM. ***$p < 0.0001$ and **$p < 0.001$ vs control. One-way ANOVA and Bonferroni *post-hoc* test.

S. AUREUS INDUCES THE SECRETION OF ADENOSINE INTO THE EXTRACELLULAR MEDIUM WHILE METAL ALLOYS TI–6AL–4V AND CO-CR-MO INCREASE SUCH CONCENTRATION

The HPLC study of extracellular nucleotide concentrations reflected a significant increase in inosine in the presence of *S. aureus* on days 3 and 5 of differentiation with IL-4 20 ng/mL ($p < 0.0001$, $n = 8$), which were reduced significantly when co-culture was performed in the presence of Ti–6Al–4V alloy on days 3 and 5 of differentiation and in the presence of Co-Cr-Mo alloy at 5 days ($p < 0.0001$, $n = 8$) (Fig. 2A). Regarding extracellular concentrations of adenosine, we observed that no significant changes occurred in the presence of IL-4 20 ng/mL alone, or *S. aureus* on days 3 or 5 of differentiation. However, both Ti–6Al–4V and Co-Cr-Mo alloys induced a significant increase in extracellular adenosine levels vs both the basal and *S. aureus* at both time points ($p < 0.0001$ vs *S. aureus*, $n = 8$) (Fig. 2B). Extracellular levels of AMP increased significantly on day 5 of differentiation in all cases ($p < 0.001$, $p < 0.0001$, $n = 8$) without significant differences between groups (Fig. 2C). No significant changes in the extracellular concentrations of ADP or ATP were found (p = ns, $n = 8$) (Fig. 2 D-E).

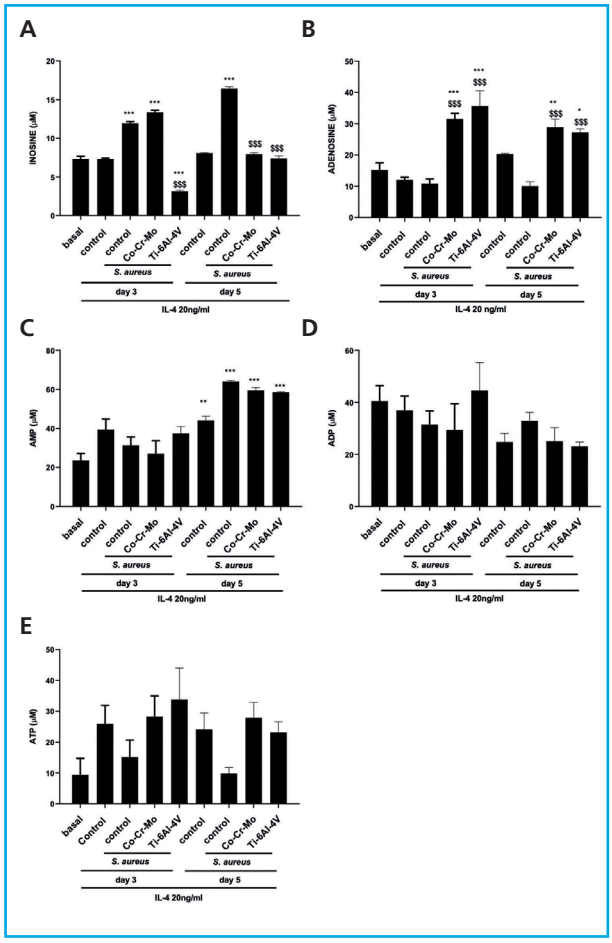

Figura 2. Nucleotide analysis via HPLC on days 3 and 5 of differentiation in the presence of adherent unviable *S. aureus* and metal alloys. A. Extracellular concentrations of inosine. B. Extracellular concentrations of adenosine. C. Extracellular concentrations of AMP. D. Extracellular concentrations of ADP. E. Extracellular concentrations of ATP. Data are presented as the mean ± SEM. ***$p < 0.0001$ vs basal; **$p < 0.001$ vs -basal; $^{\$\$\$}p < 0.0001$ vs *S. aureus*. One-way ANOVA and Bonferroni *post-hoc test.*

ADENOSINE A1 AND A2A RECEPTORS AND P2X7 RECEPTORS EXPRESSION IS MODULATED BY *S. AUREUS* AND THE PRESENCE OF METAL ALLOYS

Following the increase in extracellular adenosine levels, we then tried to understand whether purinergic receptors involved in fusogenic functions (adenosine A1 and A2A receptors and P2X7 receptors) are also involved in the formation of FBGCs in the presence *of S. aureus* and metal alloys. The expression of mRNA for adenosine A1 receptor in RAW264.7 cells increased at 3 and 5 days in the presence of 20 ng/mL IL-4 and was enhanced on day 5 of differentiation in the presence of *S. aureus* although this increase was not significant (p = ns, n = 3-4) (Fig. 3A). Although the Ti–6Al–4V alloy increased A1 receptor mRNA expression, this increase was significant on day 5 of differentiation (11120.6 ± 6630 fold change *S. aureus* + Ti–6Al–4V vs 11.7 ± 8.073 fold change *S. aureus*, $p < 0.05$, n = 3-4) (Fig. 3A). A similar trend was seen for the Cr-Co-Mo alloy, although in this case, the increased expression was not significant (p = ns, n = 3-4) (Fig. 3A).

A similar tendency was observed for adenosine A2A receptor in RAW264.7 cells. A2A receptor mRNA expression increased at 3 and 5 days in the presence of 20 ng/mL IL-4 and was enhanced on day 5 of differentiation in the presence of *S. aureus* although this increase was not significant (p = ns, n = 3-4) (Fig. 3B). Although the Ti–6Al–4V alloy increased A2A receptor mRNA expression, this increase was not significant (p = ns, n = 3-4). Furthermore, the Co-Cr-Mo alloy significantly increased A2A receptor mRNA expression on day 5 of differentiation (15114 ± 6082-fold change *S. aureus* + Co-Cr-Mo vs 8.17 ± 2.95-fold change *S. aureus*, $p < 0.05$, n = 3-4) (Fig. 3B).

The expression of the P2X7 ATP receptor does not increase significantly in the presence of IL-4 20 ng/mL, both on days 3 and 5 of differentiation. However, it does increase in the presence of *S. aureus,* with this increase being further enhanced by both metal alloys. Nevertheless, this increase was not found to be statically significant (p = ns, n = 3-4) (Fig. 3C).

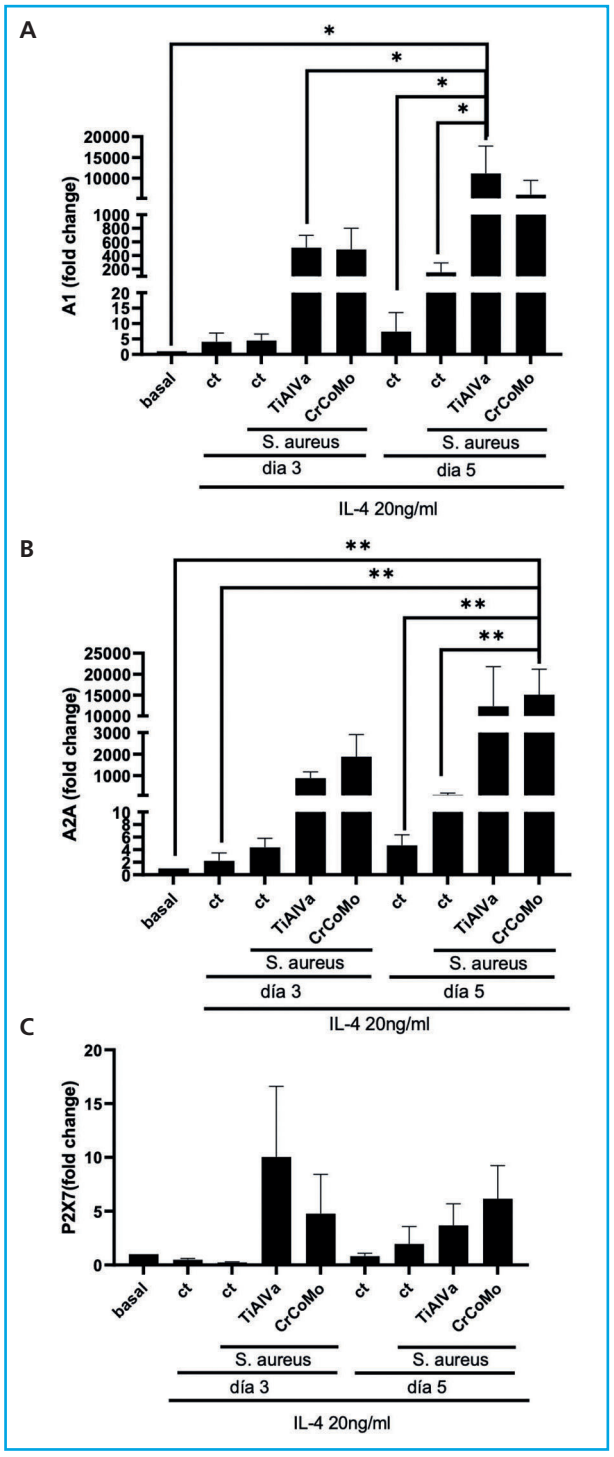

Figura 3. Purinergic receptor mRNA expression on days 3 and 5 of differentiation in the presence ofadherent unviable *S. aureus* and metal alloys. A. mRNA expression for adenosine A1 receptor in the presence of adherent unviable *S. aureus* and Ti–6Al–4V and Co-Cr-Mo alloys. B. mRNA expression for adenosine A2A receptor in the presence of adherent unviable *S. aureus* and Ti–6Al–4V and Co-Cr-Mo alloys. C. mRNA expression for P2X7 receptor in the presence of adherent unviable *S. aureus* and Ti–6Al–4V and Co-Cr-Mo alloys. Data are presented as the mean ± SEM. *$p < 0.05$. One-way ANOVA and Bonferroni *post-hoc* test.

ADENOSINE A2A RECEPTOR ACTIVATION INHIBITS *S. AUREUS*-INDUCED FBGC FORMATION

Incubation of RAW264.7 cells with the adenosine A2A receptor agonist CGS21680 1 µM, inhibited FBGCs formation induced by IL-4 20 ng/mL (reduction of 37.89 ± 1.97 % vs 100 % FBGCs in IL-4, $p < 0.0001$, $n = 6$), being reversed in the presence of the A2A receptor antagonist ZM241385 1 µM (Fig. 4). In the presence of *S. aureus*, there was an increased in FBGCs formation (increase of 70.67 ± 2.33 % vs 100 % FBGCs in IL-4, $p < 0.0001$, $n = 6$) as described above that was significantly reduced in the presence of CGS21680 1 µM (decrease of 43.17 ± 3.7 % vs IL-4 and *S. aureus*, $p < 0.0001$, $n = 6$) which is also reversed in the presence of ZM241385 1 µM (Fig. 4).

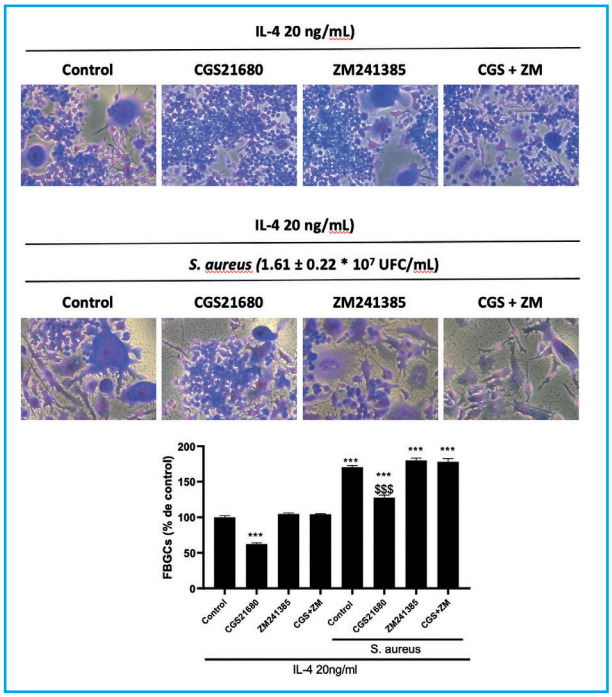

Figura 4. The formation of FBGCs in the presence of *S. aureus* is decreased by the adenosine A2A receptor agonist CGS21680. Panoptic staining representative images and quantification for FBGCs formation. RAW264.7 cells treated with IL-4 20 ng/mL and adenosine A2A receptor agonist CGS21680 1 µM and antagonist ZM241385 1 µM alone or in the presence of adherent unviable *S. aureus*. All images were taken at 40x magnification. Data are presented as the mean ± SEM. ***$p < 0.0001$ vs control; $^{\$\$\$}p < 0.0001$ vs *S. aureus*. One-way ANOVA and Bonferroni *post-hoc* test.

S. AUREUS AND METAL ALLOYS SLIGHTLY CHANGE THE CYTOKINES PROLIFE SECRETION OF RAW264.7 CELLS

Afterwards, we tried to identify changes in cytokine secretion due to presence of *S. aureus* and metal al-

loys. For this purpose, we performed a cytokine array that measures 20 cytokines and chemokines (Table II). When we analyzed the expression of pro-inflammatory cytokines, we found that the presence of *S. aureus* only increased the expression of IL-6 and TNFα vs both with basal conditions and control conditions in the presence of IL-4 20 ng/mL (137.2 ± 23.12 pg/mL *S. aureus* vs 2.28 ± 1.23 pg/mL IL-4 for IL-6; and 781.1 ± 289.9 pg/mL *S. aureus* vs 7.051 ± 3.287 pg/mL IL-4 for TNFα, $p < 0.0001$ and $p < 0.05$ respectively, $n = 5$-6) (Table II). When RAW264.7 cells were differentiated in the presence of IL-4 20 ng/mL and the Co-Cr-Mo alloy, no differences in pro-inflammatory cytokines were observed vs control conditions in the presence of IL-4 20 ng/mL (Table II). However, when RAW264.7 cells were differentiated in the presence of *S. aureus* and the Co-Cr-Mo alloy, we observed a significant increase in IL-1α, IL-1β and IL-6 expression vs all conditions (336.6 ± 139.5 pg/mL *S. aureus* + Co-Cr-Mo vs 22.98 ± 10.5 pg/mL *S. aureus* for IL-1α; 114.5 ± 23.21 pg/mL *S. aureus* + Co-Cr-Mo vs 2.636 ± 1.895 pg/mL *S. aureus* for IL-1β; and 925.9 ± 283.621 pg/mL *S. aureus* + Co-Cr-Mo vs 137.2 ± 23.12 pg/mL *S. aureus* for IL-6, $p < 0.05$, $p < 0.0001$ and $p < 0.0001$ respectively, $n = 5$-6) (Table II). In a similar way, when RAW264.7 cells were differentiated in the presence of IL-4 20 ng/mL and the Ti–6Al–4V alloy, no differences in pro-inflammatory cytokines were observed vs control conditions in the presence of IL-4 20 ng/mL (Table II). Moreover, when RAW264.7 cells were differentiated in the presence of *S. aureus* and the Ti–6Al–4V alloy, we observed a significant increase in IL-1β and IL-6 expression when vs all conditions (209.5 ± 52,88 pg/mL *S. aureus* + Ti–6Al–4V vs 2.636 ± 1.895 pg/mL *S. aureus* for IL-1β; and 3366 ± 818.3 pg/mL *S. aureus* + Ti–6Al–4V vs 137.2 ± 23.12 pg/mL *S. aureus* for IL-6, $p < 0.0001$ and $p < 0.0001$ respectively, $n = 5$-6) (Table II).

No changes were observed among conditions in any anti-inflammatory cytokine (Table II).

RANTES, a classical chemotactic cytokine also known as CCL5, was increased in the presence of *S. aureus* (528.3 ± 66.47 pg/mL *S. aureus* vs 199.8 ± 37.12 pg/mL IL-4, $p < 0.001$, $n = 5$-6) and enhanced when co-culture was performed in the presence of metal alloys (609.6 ± 81.64 pg/mL *S. aureus* + Co-Cr-Mo vs 528.3 ± 66.47 pg/mL *S. aureus* and 994.4 ± 185,4 pg/mL *S. aureus* + Ti–6Al–4V vs 528,3 ± 66,47 pg/mL *S. aureus*, $p < 0.001$ and $p < 0.0001$ respectively, $n = 5$-6) (Table II).

Finally, we found that the presence of IL-4 20 ng/mL induced an increased expression of VEGF (360.8 ± 44.25 pg/mL IL4 vs 82.94 ± 46.62 pg/mL basal, $p < 0.0001$, $n = 5$-6) that was not significantly reduced in the presence of *S. aureus* but was significantly increased in the presence of both IL-4 and the Co-Cr-Mo alloy (669.3 ± 144.5 pg/mL Co-Cr-Mo vs 82.94 ± 46,62 pg/mL basal, $p < 0.0001$, $n = 5$-6) (Table II).

DISCUSSION

In this manuscript we have demonstrated that the presence of Ti–6Al–4V and Co-Cr-Mo alloys required purinergic system activation in macrophages to inhibit FBGCs formation induced by adhered unviable *S. aureus*.

As we can conclude from our data, adhered unviable *S. aureus* can form FBGCs although non-infectious activity. Although the use of non-viable *S. aureus* represents a limitation of our study, it is worth noting that during *S. aureus* biofilm formation bacterial lysis plays a key role (24), so this study may reveal what role these non-viable cells play in the development of FBGCs. *S. aureus* can attach to biotic surfaces through electrostatic and hydrophobic interactions under static conditions, even if it is not viable (25). These adhered unviable staphylococci which are not forming a biofilm will be detected by the Toll-like receptors and induce the phagocytic cell activation (26). Macrophage fusion to form a FBGC occurs in response to tissue injury and the presence of any biomaterial (7), whose physical features (ie, substrate stiffness, topography, and surface chemistry) will determine such response. Interestingly, this foreign body response is also regulated by Toll-like receptors (27). Our results illustrate that unviable *S. aureus* can induce the formation of FBGCs. Furthermore, it is plausible that the presence of metal alloys Ti–6Al–4V and Co-Cr-Mo would be necessary for the inhibition of that formation of FBGCs even in the presence of unviable *S. aureus*.

Fibrotic encapsulation emerged as a crucial tissue response to foreign objects inadvertently becoming lodged in the body. The immune capacity to successfully block access to the rest of the body by fibrotic encapsulation is pivotal since these foreign items pose numerous hazards, including the potential to spread infection (28). If fibrotic encapsulation were to take place on the *S. aureus*-infected implant, the infection foci would have access to fewer nutrients from the periprosthetic tissues. Hence, the inhibition of the development of FBGCs by those metal alloys may inhibit the fibrous encapsulation of these staphylococci-covered alloys (29), thus favoring the maintenance of the staphylococcal infection which would not have any fibrotic capsule impairing the arrival of nutrients into their immediate environment.

HPLC analysis showed increased adenosine extracellular levels induced by metal alloys that were not exert neither by IL-4 alone nor in combination with adhered unviable *S. aureus*. This adenosine increased correlates with the expression of adenosine receptors showed. Although both metal alloys increased in a similar manner adenosine levels, and both have the same trend in adenosine A1 and A2A receptors mRNA increased, Ti–6Al–4V only significantly increased mRNA levels for adenosine A1 receptor meanwhile Co-Cr-Mo only significantly induced adenosine A2A receptor.

Table II. Inflammatory cytokines level measured using the mouse cytokine array Q1

Cytokines (pg/mL)	Basal	Control	*S. aureus*	Cr-Co-Mo	*S. aureus* + CrCoMo	Ti–6Al–4V	*S. aureus* + Ti–6Al4V
Pro-inflammatory							
IFNγ	12.33 ± 9.696	12.54 ± 2.968	23.58 ± 14.54	31.18 ± 16.48	6.729 ± 3.414	15.01 ± 3.93	63.93 ± 30.65
IL-1α	0 ± 0	0.1422 ± 0.08105	22.98 ± 10.5	5.4793.424	336.6 ± 139.5*	83.27 ± 59.56	581.82 ± 281.9
IL-1β	0 ± 0	0 ± 0	2.636 ± 1.895	15.11 ± 5.793	114.5 ± 23.21***	26.07 ± 10.79	209.5 ± 52.88***
IL-2	0 ± 0	0.1456 ± 0.1018	0.4513 ± 0.289	0.3433 ± 0.2103	0.2461 ± 0.1813	0.1256 ± 0.08059	5.51 ± 3.437
IL-3	0 ± 0	0 ± 0	0 ± 0	0.0008575 ± 0.0008575	0 ± 0	0.0008473 ± 0.0008473	0.06079 ± 0.06079
L-6	0.08741 ± 0.08741	2.282 ± 1.226***	137.2 ± 23.12***	18.17 ± 8.755	925.9 ± 283.6***	62.05 ± 20.41	3366 ± 818.3***
IL-12	36.8 ± 27.04	22.1 ± 13.84	22.2 ± 22.2	53.17 ± 34.78	1.024 ± 0.9375	50.31 ± 34.34	57.24 ± 25.82
IL-17	18.81 ± 12.08	45.7 ± 21.8	49.75 ± 32.69	57.45 ± 32.05	36.84 ± 23.44	23.41 ± 15.74	44.41 ± 43.1
TNFα	28.1 ± 10.07	7.051 ± 3.287	781.1 ± 289.9*	27.71 ± 19.72	638.8 ± 258.3	206.1 ± 108.9	839 ± 282.4
Anti-inflammatory							
IL-4	0 ± 0	990.3 ± 417.9	839.1 ± 313.3	1243 ± 553.6	1043 ± 448	1055 ± 576.4	1053 ± 382.2
IL-5	12.08 ± 12.08	11.57 ± 6.139	6.548 ± 3.407	68.58 ± 45.6	4.693 ± 2.211	102.2 ± 64.11	106.2 ± 48.8
IL-9	32.74 ± 32.74	0 ± 0	72.46 ± 53.64	68.58 ± 68.58	2.842 ± 2.842	43.19 ± 41.89	294.2 ± 135.7
IL-10	9.099 ± 6.118	26.76 ± 17.81	26.94 ± 13.94	40.2 ± 18.28	21.57 ± 12.59	31.86 ± 10.41	205.7 ± 124.8
IL-13	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.04131 ± 0.04131	79.55 ± 78.01
Chemokines (pg/mL)							
KC	0 ± 0	0.2814 ± 0.2259	0.05176 ± 0.05176	1.039 ± 0.7841	0.2598 ± 0.2598	0.01222 ± 0.01222	49.18 ± 32.2
RANTES	184.2 ± 51.74	199.8 ± 37.12	528.3 ± 66.47**	312.7 ± 92.16	609.6 ± 81.64**	230.2 ± 109.4	994.4 ± 185.4***
Monocyte chemoattractant (pg/mL)							
MCP-1	19344 ± 10709	24237 ± 11396	70425 ± 37955	196673 ± 138323	101614 ± 55319	8901 ± 5512	57306 ± 37478
Differentiation and proliferation of macrophages (pg/mL)							
GM-CSF	0 ± 0	0.4957 ± 0.4957	0.7988 ± 0.7988	16.03 ± 11.65	0.4237 ± 0.4237	0.3985 ± 0.3985	33.38 ± 18.46
M-CSF	0 ± 0	0.03008 ± 0.02416	0 ± 0	0.01709 ± 0.0115	0 ± 0	0 ± 0	1.844 ± 1.839
Vasculogenesis and angiogenesis (pg/mL)							
VEGF	82.94 ± 46.62	360.8 ± 44.25***	24.77 ± 12.02	669.3 ± 144.5***	177.4 ± 32.91	299.6 ± 132.7	457.2 ± 172.1

*Cytokines and chemokines in pg/mL. Data are express as mean ± SEM. *p < 0.05, **p < 0.001, and ***p < 0.0001. One-way ANOVA and Bonferroni post-hoc test.*

None of the conditions induce P2X7 changes, in correlation with no changes of ATP levels. Considering that both FBGCs and osteoclasts share some mechanism during their formation process (10), it is plausible that adenosine receptors have a similar role in both FBGCS and osteoclast formation. It has been extensively demonstrated that adenosine A2A receptor inhibits osteoclast formation (14,15). The increase in this receptor together with the rise in adenosine levels induced by metal alloys indicate that similar role might be award to this receptor in FGBCs formation, primary in the presence of Co-Cr-Mo alloys.

On the other hand, it has been demonstrated that adenosine A1 receptors are involved in fusion of human peripheral blood monocytes into giant cells although ith as not been fully established (30). It is known that adenosine A1 receptor induces osteoclast differenti-

ation (13), and blockade or deletion of the receptor suppresses osteoclast differentiation *in vitro* and *in vivo* (31). Furthermore, it has been demonstrated that adenosine A1 receptor on both primary murine and human osteoclast precursors is constitutively active, with selective antagonists working as an inverse agonists at A1 receptor (32), which might be the reason why changes in adenosine A1 receptor mRNA expression in IL-4 and *S. aureus* alone groups vs basal conditions are not significant.

Although the increase in A1 receptor in the presence of metal alloys needs to be address better, such an increase might be a compensatory mechanism induced by *S. aureus* as it has been observed that A1 receptor stimulation is essential for the formation of giant cells *in vitro* in the syncytia in giant cell arteritis, a characteristic where inflammation is crucial (33). Our data indicate that the presence of metal alloys induces a significant increase in IL-1β and IL-6, 2 key pro-inflammatory cytokines. This increase may be involved in the rise of the adenosine A1 receptor. However, the inflammatory role of adenosine A1 receptor is controversial. As it has been observed in some scenarios, A1 receptor can induce anti-inflammatory effects, including renal ischemia reperfusion, lungs (where the absence of adenosine A1 receptor induce leukocyte migration and increased cytokines), and sepsis where A1KO mice exhibit a higher degree of renal dysfunction and higher release of pro-inflammatory cytokines (34). These data indicate that the presence of endogenous A1 receptor is necessary to protect vs exacerbation of the disease and organ dysfunction. It is plausible that same as it occurs in sepsis, the imbalance in inflammation induced by *S. aureus* promotes the activation of A1 receptor by metal alloys in our system to counteract the hyper-inflammatory scenario, being in this situation not only the adenosine A2A receptor—the only anti-inflammatory receptor—but also the A1 receptor. Therefore, more data are needed to try to understand metal alloys induced A1 receptor expression in this system.

Finally, one of the limitations of this study was the impossibility of using live *S. aureus* bacteria in our assays. Although different bacterial infection assays were performed (data not shown), the results obtained were erratic and the amount of FBGCs remaining adhered after infection was too small to perform the assays conducted in this study. It would be convenient to conduct experiments with viable bacteria, with validation of the appropriate moments of infection, co-culture methods (adherence or non-adherence), etc.

In conclusion, the increased levels of adenosine would be responsible for the inhibition of cell fusion and the following reduction of *S. aureus*-induced FBGCs. Also relevant is the activation of the adenosine A2A receptor induced by the presence of Co-Cr-Mo alloys.

ACKNOWLEDGMENTS

This work was supported by grants from Becas FEIOMM INVESTIGACIÓN 2021 and Instituto de Salud Carlos III through the "Miguel Servet" program (CP15/00053, CPII20/00017), co-funded by Fondo Europeo de Desarrollo Regional (FEDER), and a research grant from the Spanish Instituto de Salud Carlos III (PI19/00744, PI22/00347).

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Revista de Osteoporosis y Metabolismo Mineral

Original

Effect of deficient vitamin D levels on muscular activity and vascular health in an experimental model

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Abstract

Introduction: previous studies show that adequate levels of calcidiol are associated with greater muscle strength, maintenance of daily activities and less progression of aortic calcification. The objective of this study was to assess the effect of vitamin D deficiency on muscular activity and vascular health in an experimental model.

Material and methods: 18-month-old FVB/N mice were used. One group received a diet without vitamin D (deficient group, $n = 20$) and another group received a normal diet (control group, $n = 17$) for 8 weeks. To measure muscular activity, a wooden rod fixed to a table with a mark 10 cm from the supported end was used to indicate the finish line. Mice were placed on the "open" end of the rod with their backs to the target. After a previous training process, the animals were evaluated three times measuring two parameters: orientation time (time necessary to turn 180° from the initial position and look towards the supported end) and transition time (time necessary to reach the goal). At sacrifice, blood and tissues were removed.

Results: calcidiol levels were higher in the control group (23.3 ± 3.9 *vs* 12.7 ± 3.1 ng/mL, $p < 0.001$). Orientation times (43 ± 46 *vs* 15 ± 21 seconds, $p = 0.020$) and transition times (62 ± 51 *vs* 31 ± 37 seconds, $p = 0.041$) were much higher in the deficient group compared to the control group. At the aortic level, the gene expression of α-actin and miR-145 were highly compromised in the deficient group. Runx2 expression was not altered.

Conclusions: these experimental results confirm previous clinical results where maintaining adequate levels of vitamin D prevents the loss of muscular functionality and vascular damage.

Keywords:
Vitamin D.
Muscular activity.
Vascular health.

Received: 28/08/2024 • Accepted: 18/11/2024

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

Artificial Intelligence: the authors declare that they did not use artificial intelligence (AI) or any AI-based tools for the drafting of this article.

Martín Vírgala J, Sosa Calleja P, Fernández Villabrille S, Martín Carro B, Naves Mendívil L, González García N, Alonso Montes C, Panizo García S, Carrillo López N, Ruiz Torres MP, Dusso A, Naves Díaz M. Effect of deficient vitamin D levels on muscular activity and vascular health in an experimental model. Rev Osteoporos Metab Miner 2024;16(4):133-139

DOI: 10.20960/RevOsteoporosMetabMiner.00060

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INTRODUCTION

The endocrine system of vitamin D modulates the expression of more than 3 % of all genes in the body, thus regulating various physiological processes in other organs and systems, such as the muscle (1). In recent years, there has been an emphasis on maintaining an adequate vitamin D status to optimize muscle strength and reduce falls and fractures in the elderly population (2-4).

Vitamin D stimulates the absorption of calcium in the intestine and maintains serum calcium levels required for muscle function (5). Several in vivo studies suggest the role of vitamin D in regulating muscle mass and function. Observational studies demonstrate that vitamin D deficiency in elderly individuals is associated with reduced muscle mass and strength (6-8), lower physical performance (6,9), and an increased risk of falls (10). Furthermore, a meta-analysis of 17 clinical trials showed that vitamin D supplementation in subjects with baseline calcidiol levels < 10 ng/mL had a positive effect on hip muscle strength (11). These studies suggest that vitamin D may affect muscle mass and function; however, it remains unclear whether vitamin D plays a direct or indirect role.

In recent years, increasing attention has been given to the local conversion of calcidiol to calcitriol, the most active metabolite of vitamin D, which is synthesized mainly in the kidney through its precursor, calcidiol (5). This local synthesis has been demonstrated in several cell types, such as osteoblasts (12-15) and monocytes (16), reinforcing the importance of maintaining adequate calcidiol levels in the body.

The vitamin D endocrine system also regulates the cardiovascular system (17). In fact, data from our group, in a cohort of an unselected general population, have shown that adequate calcidiol levels are associated not only with greater muscle strength and maintenance of daily activities but also with less progression of aortic calcification (18,19).

Therefore, the objective of this study was to evaluate the effect of vitamin D deficiency on motor activity and vascular health in an experimental model. This type of study allows for greater precision in limiting and controlling potential biases inherent in epidemiological studies.

MATERIALS AND METHODS

Male and female FVB/N mice, 18 months of age, were used. One group received a vitamin D-deficient diet (Teklad 2014, Vitamin D omitted, Envigo, Spain) (deficient group, *n* = 20), while the other remained on a normal diet (Teklad 2014, standard version, Envigo) (control group, *n* = 17) for 8 weeks.

To induce the expression of CYP24A1 or renal 1-α hydroxylase and accelerate the catabolism of endogenous calcidiol and calcitriol reserves, the animals received intraperitoneal injections of 3 ng of 19-nor-1,25-dihydroxyvitamin D2 (paricalcitol; Zemplar, kindly provided by Abbott, now AbbVie, Lake Bluff, IL, USA) on days 1, 3, 5, 8, 10, and 12, following a protocol used by other authors to accelerate vitamin D catabolism (20).

At the end of the last week of treatment with the deficient or normal diet, motor activity was measured using a wooden rod (60 cm long and 28 mm in diameter) fixed to a table 60 cm above a cushioned surface, with a mark 10 cm from the supported end to indicate the finish line (21). Mice were placed at the "open" end of the rod, facing away from the finish line (Fig. 1). Two weeks before conducting the motor activity study, a training process was conducted to acclimate the animals to the rod, with a treat placed at the finish line to reward their effort. During the study, animals were evaluated three times, measuring two parameters: orientation time (time needed to turn 180° from the initial position to face the supported end) and transition time (time required to reach the finish line). If a mouse fell twice during the orientation or transition times, 25 seconds were added to the time recorded. Each mouse had a total of 2 minutes to complete the test. At sacrifice, blood and aortic tissue were collected and stored at -80 °C until processing.

Serum biochemical determinations were performed using specific Quanti-Chrom™ kits (Bioassay Systems, Hayward, CA, USA) for BUN, Ca, and P. For other biochemical markers, specific ELISA kits were used: PTH (Immutopics, Inc., San Clemente, CA, USA), intact FGF23 (Immutopics), and calcidiol (Immunodiagnostic Systems Ltd, Scottsdale, AZ, USA).

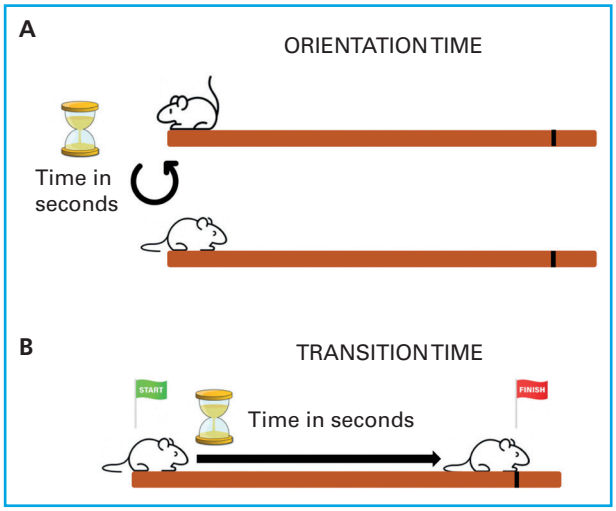

Figure 1. Diagram representing motor activity. A. Orientation time is defined as the time (in seconds) it takes for the animal to turn around on the rod to change the direction of movement. B. Transition time represents the time (in seconds) the animal takes to traverse the rod to the finish line.

The aortas of the animals were not included in paraffin to assess calcifications, as mice are not a good model for observing calcifications (22) unless they are ApoE mutants (23). Additionally, in our case, the mice had normal renal function, so finding calcifications at this level, even in aged mice, was unlikely.

Total RNA from kidney and aorta was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA). Two µg of total RNA was reverse-transcribed into cDNA using the High-Capacity Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Gene expression in the aorta (α-actin, Runx2, and microRNA (miR)-145) and kidney (1-α hydroxylase and 25-α hydroxylase) was assessed using qPCR with pre-designed assays (Applied Biosystems) on a Stratagene Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). All reactions were performed in triplicate. Relative gene expression was quantified using the ΔΔCT method, with GAPDH and U6 as housekeeping genes for microRNA, and expressed in relative units (RU) (24).

STATISTICAL ANALYSIS

Data analysis was performed using SPSS version 25.0 for Windows. Quantitative variables were analyzed using Student's t-test with a significance threshold of $p < 0.05$ to highlight statistically significant differences.

RESULTS

At biochemical level, there were no differences between mice fed a vitamin D-deficient diet and those fed a normal vitamin D diet in renal function measured by BUN, or in serum levels of calcium, phosphorus, PTH, or FGF23 (Table I). As expected, calcidiol levels were significantly higher in the group receiving the normal vitamin D diet compared to those on the deficient diet (23.3 ± 3.9 vs 12.7 ± 3.1 ng/mL, p < 0.001) (Table I).

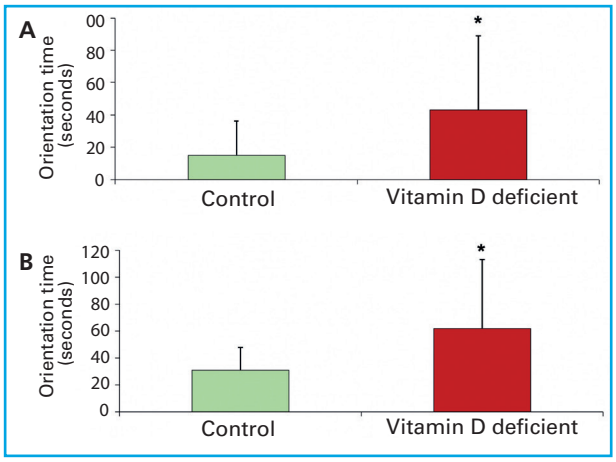

Figure 2. Motor activity measurements of orientation (A) and transition times (B). Data show measurements taken on a wooden rod in groups of mice fed a vitamin D-deficient or normal diet. *$p < 0.05$ *vs* the control group (normal vitamin D diet group).

When studying muscle activity parameters, it was observed that orientation (43 ± 46 vs 15 ± 21 seconds, $p = 0.020$) and transition times (62 ± 51 vs 31 ± 37 seconds, $p = 0.041$) were significantly higher in the group on the vitamin D-deficient diet vs the normal diet group (Fig. 2). The minimum time required for orientation was 2 seconds, and for transition, it was 6 seconds. In both cases, the maximum time was 120 seconds. In the vitamin D-deficient group, 35 % of mice were unable to complete the task vs 17.6 % in the normal diet group.

At aortic level, gene expression of α-actin, a vascular phenotype marker, was markedly impaired in the vitamin D-deficient group (2.05 ± 1.48 vs 0.98 ± 0.51 RU, $p = 0.041$). Expression of miR-145, the key microRNA regulating contractile phenotype, also showed a similar pattern to α-actin, with a significant reduction in its expression in vitamin D-deficient mice (1.70 ± 1.72 vs 0.76 ± 0.34 RU, $p = 0.048$). Runx2 expression remained unaltered (Fig. 3).

Table I. Biochemical markers in the experimental study of mice fed a normal or vitamin D-deficient diet

Biochemical markers	Normal diet group ($n = 17$)	Deficient diet group ($n = 20$)	*p*-value
BUN (mg/dL)	23.2 ± 4.5	22.7 ± 3.8	0.846
Calcium (mg/dL)	9.1 ± 0.6	8.9 ± 0.8	0.563
Phosphorus (mg/dL)	5.3 ± 1.2	5.4 ± 1.3	0.929
PTH (pg/mL)	173 ± 58	202 ± 104	0.513
FGF23 (pg/mL)	239 ± 124	288 ± 58	0.398
Calcidiol (ng/mL)	23.3 ± 3.9	12.7 ± 3.1	*< 0.001*

At renal level, gene expression of 1-α hydroxylase and 25-α hydroxylase showed no statistically significant differences between vitamin D-deficient and non-deficient animals. However, there was a 39 % reduction in 1-α hydroxylase gene expression in the vitamin D-deficient group (1.83 ± 2.19 vs 3.00 ± 4.77 RU, $p = 0.413$). This reduction was 23 % for 25-α hydroxylase (1.60 ± 2.39 vs 2.08 ± 2.00 RU, $p = 0.586$).

DISCUSSION

These experimental results confirm previous clinical findings that maintaining adequate vitamin D levels prevents motor function loss and vascular damage.

First, in aged mice, it was shown that a lack of vitamin D affects mobility, including orientation and completing specific tasks. Several years ago, our group published an article in the general population showing that vitamin D deficiency was associated with reduced handgrip strength and greater difficulty in performing daily activities. This appears to be corroborated by this experimental study (18).

Vitamin D deficiency is common in older adults and may contribute to muscle deterioration through sarcopenia (25,26). This results in devastating effects, increasing care needs and morbidity/mortality among older individuals (25). Additionally, older adults face a higher risk of vitamin D deficiency due to age-related factors such as decreased synthesis or reduced sunlight exposure (27). Although a recent meta-analysis questions the utility of vitamin D supplements for reducing fall risk, bone mineral density loss, and fractures, there is ample evidence of the importance of vitamin D for muscle and bone health (28).

While there is debate about whether the vitamin D receptor (VDR) is expressed in muscle, some authors have found significantly higher VDR levels in young mice muscle, supporting a pleiotropic effect in this tissue (29-31). Moreover, VDR expression in muscle is known to decline with age (32,33), making the musculoskeletal system more vulnerable to low vitamin D levels in the elderly. Skeletal muscle appears to act as a significant storage site for vitamin D, which may diffuse back into circulation or to adjacent areas under specific signals (34,35).

We were unable to detect a reduction in renal 1-α hydroxylase expression in vitamin D-deficient mice, though there was a trend toward reduction, likely due to the lack of calcidiol substrate for conversion to its active form, calcitriol. It should be noted that muscle expresses the 1-α hydroxylase enzyme, facilitating local calcitriol synthesis (30,36,37), particularly in mice with adequate calcidiol levels.

Although mice are not ideal models for studying vascular calcification due to their resistance to calcification, molecular alterations indicating vascular changes were observed. The significant reductions (over 50 %) in α-actin expression, the most specific marker of contractile phenotype, in vitamin D-deficient mice suggest potential signs of a shift from vascular to osteogenic phenotype. However, Runx2 levels were not elevated. Interestingly, miR-145 expression, the key regulator of vascular phenotype, was markedly reduced. Recent findings from our group suggest that the loss of miR-145 precedes α-actin reductions or intracellular calcium deposits in the vascular-to-osteogenic phenotype transition (38).

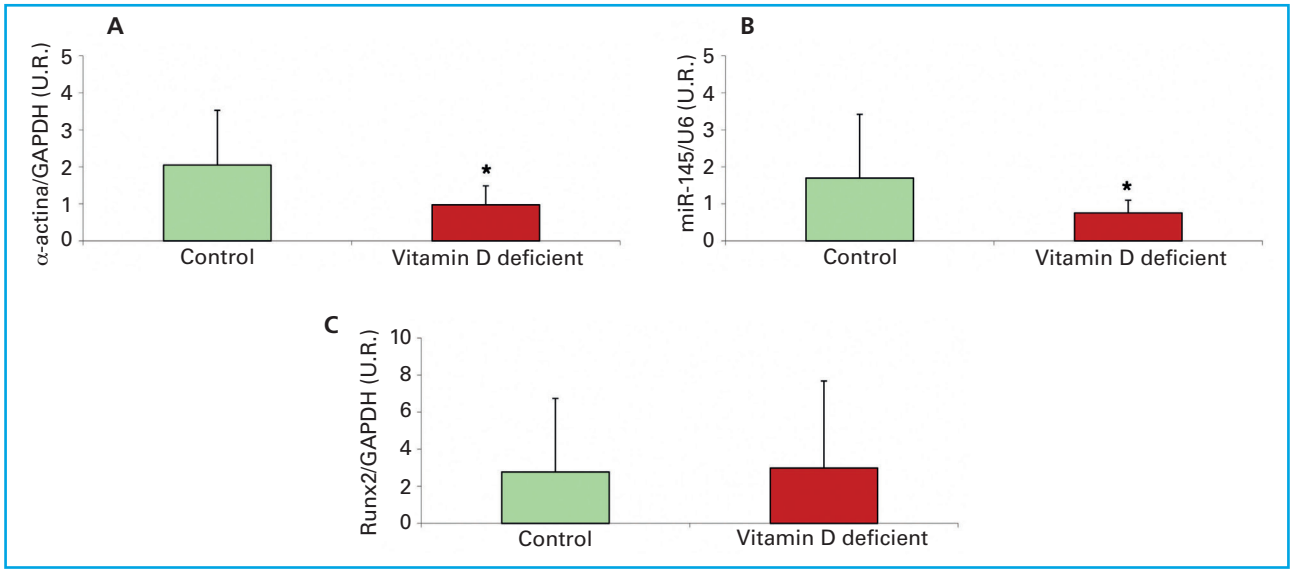

Figure 3. Relative gene expression in the aortas of mice fed normal and vitamin D-deficient diets. A. α-actin expression. B. miR-145 expression. C. Runx2 expression. *$p < 0.05$ vs the control group (normal vitamin D diet group).

Thus, maintaining adequate miR-145 levels appears essential for vascular health. Among the inducers of miR-145 expression is vitamin D, which mediates its antiproliferative and gene-regulating effects, potentially benefiting the prognosis and development of therapeutic strategies for gastric cancer (39). In a recent study, our group demonstrated that vitamin D administration prevented reductions in miR-145 and α-actin in vascular smooth muscle cells induced by uremia, thereby reducing vascular contractility and osteogenic differentiation alterations (40).

As other authors have pointed out (41), several mechanisms explain the link between vitamin D deficiency and cardiovascular diseases. Vitamin D deficiency stimulates PTH gene transcription, promoting myocyte hypertrophy and inflammatory mechanisms involving vascular smooth muscle cells. The vitamin D endocrine system also suppresses inflammatory processes, a key pathogenic mechanism in atherosclerosis, and exerts antiproliferative effects, reducing myocardial hypertrophy and damage (41).

It is essential to consider the challenges of extrapolating rodent model results to humans. However, these findings align with clinical studies while controlling confounding factors inherent to patient studies. Additionally, mice are not optimal models for studying vascular calcification due to their resistance to it. Nonetheless, the observed vascular alterations may indicate an initial vascular phenotype change leading to calcification.

This study shows that vitamin D deficiency in an aged mouse model impairs motor functionality and induces vascular phenotype changes. These vascular alterations include a marked loss of α-actin, the primary contractile protein in vascular smooth muscle cells, and miR-145, the main regulator of vascular phenotype.

ACKNOWLEDGMENTS

This study was funded by Instituto de Salud Carlos III (ISCIII)-Fondo de Investigación Sanitaria (PI16/00637, PI17/00715, PI17/00384, and PI19/00532), the European Regional Development Fund (FEDER), the Science, Technology, and Innovation Plans 2013-2017 and 2018-2022 of the Principality of Asturias (GRUPIN14-028, IDI-2018-000152, IDI-2021-000080, IDE/2024/000706), Fundación Renal Íñigo Álvarez de Toledo (FRIAT), and the ISCIII REDinREN network (RD16/0009/0017 and RD16/0009/0018) and RICORS2040 (Kidney Disease; RD21/0005/0019 and RD21/0005/0023). Sara Panizo García and Natalia Carrillo López are Miguel Servet postdoctoral researchers. Cristina Alonso Montes is a postdoctoral researcher at Instituto de Investigación Sanitaria del Principado de Asturias. Julia Martín Vírgala and Beatriz Martín Caro are postdoctoral researchers under RICORS2040 (Kidney Disease; RD21/0005/0019). Sara Fernández Villabrille is a Severo Ochoa predoctoral researcher for the Principality of Asturias. Nerea González García is a PFIS predoctoral researcher associated with project PI22/00195.

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Revista de Osteoporosis y Metabolismo Mineral

Original

Proteomics applied to the study of vascular calcification in chronic kidney disease

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Abstract

Introduction: vascular calcification (VC) is associated with increased mortality in the general population, as well as in patients with chronic kidney disease, in whom prevalence is much higher. The need for effective and early diagnosis of VC to improve preventive and prognostic strategies has driven research on biomarkers. The aim of this study is to examine the differential expression of proteins associated with the VC process using proteomics techniques.

Materials and methods: vascular smooth muscle cells were cultured under non-calcifying and calcifying conditions. Differential protein expression was performed using 2D-DIGE and LC-ESI-MS/MS, and identification was conducted using with the MASCOT search engine.

Results: after 6 days of culture, a total of 121 protein spots with differential expression were detected. A total 21 out of all these proteins were identified in 24 spots. In the cells cultured in the calcifying medium, a total of 4 showed a significantly increased expression; collagen type I exhibited the greatest change (3.49 times) vs those cultured in a non-calcifying medium. Other muscle and structural proteins showed a decrease in their expression. Furthermore, changes were reported in the expression of nucleobindin-1 and endoplasmin, which had not previously been associated with VC.

Conclusion: the results confirmed the reduction in the expression of typical muscle and cytoskeletal proteins during VC. Additionally, changes in the expression of proteins that had not previously been associated with VC were identified, and these may be involved in the process.

Keywords:
Vascular calcification.
Chronic kidney disease.
Proteomics.
2D-DIGE.

Received: 13/09/2024 • Aceptado: 02/12/2024

Authors' contributions: Cristina Alonso Montes and Jorge B. Cannata-Andía are the corresponding authors of this article. Covadonga Quirós Caso and Natalia Carrillo López have contributed equally as first authors. José Luis Fernández-Martín and Jorge B. Cannata-Andía have contributed equally as senior authors.

Funding or financial support: this original article has been funded by a FEIOMM Basic Research Grant 2021. This work has been partially funded by the RETIC REDinREN of Instituto de Salud Carlos III (ISCIII) (RD06/0016/1013, RD12/0021/0023, RD16/0009/0017), the Cooperative Health-Oriented Research Network RICORS2040 (RD21/0005/0019 funded by the European Union's NextGenerationEU through the Recovery and Resilience Facility [RRF]), ISCIII projects (PI11-00667, ICI14/00107, PI17/00384, PI20/00633, and PI24/01785 cofunded by the European Union), the National R&D&I Plan 2008-2011, the State R&D&I Plan 2013-2016, the European Regional Development Fund (ERDF), the Science, Technology and Innovation Plan 2013-2017 and 2018-2022 of the Principality of Asturias (GRUPIN14-028, IDI-2018-000152, IDI/2021/000080), the Fundación Renal Íñigo Álvarez de Toledo (FRIAT), and the FEIOMM Basic Research Grant 2021.

Acknowledgments: we wish to thank Asociación para la Lucha Contra las Enfermedades Renales del Principado de Asturias (ALCER) and Asociación de Diabetes del Principado de Asturias (ASDIPAS).

Conflicts of interest: Jorge B. Cannata-Andía declared to have received institutional and personal grants from FAES, SPAFARMA, and MabXience. The remaining authors declared no conflicts of interest whatsoever.

Artificial Intelligence: the authors declare that they did not use artificial intelligence (AI) or any AI-based tools for the drafting of this article.

Quirós-Caso C, Carrillo-López N, Fernández-Martín JL, Alonso-Montes C, Cannata-Andía JB. Proteomics applied to the study of vascular calcification in chronic kidney disease. Rev Osteoporos Metab Miner 2024;16(4):140-148

DOI: 10.20960/RevOsteoporosMetabMiner.00061

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INTRODUCTION

In patients with chronic kidney disease (CKD), the mechanisms regulating Ca and phosphorus homeostasis are compromised, leading to what is known as "Chronic Kidney Disease-Mineral and Bone Disorders" (CKD-MBD), which includes vascular calcification (VC) (1).

VC is associated with an increased risk of mortality in the general population (2) and especially in patients with CKD (3,4), in whom it is more prevalent than in individuals of the same age with preserved renal function (5,6).

The high morbidity and mortality of CKD have been attributed to both traditional and non-traditional risk factors. As in the general population, traditional risk factors (smoking, hypertension, diabetes, and male sex) are largely responsible for the progression of VC. However, these factors cannot explain the high prevalence of cardiovascular complications in CKD patients (7). Among non-traditional factors, hyperphosphatemia is one of the most studied, being related to the increased VC and mortality in CKD patients (8,9).

VC occurs through a complex, active, and regulated process involving different mechanisms (10-12). Several studies have been published to date that have advanced knowledge on the underlying mechanisms of the VC process (7,10). However, the inability to detect VC early has driven research into potential new biomarkers that could be used for early diagnosis of VC and improve preventive and prognostic strategies. Mass spectrometry applied to proteomics studies allows for the characterization of the proteomic profile of a biological sample rapidly at a specific point in time. Therefore, the main objective of this work was to study the differential expression of proteins associated with the VC process using proteomics techniques, employing an in vitro VC model with vascular smooth muscle cells (VSMCs).

MATERIALS AND METHODS

CELL CULTURE AND CALCIFICATION CONDITIONS

The A7r5 cell line from rat aorta (ATCC CRL-1444™) was used as VSMCs. The cells were cultured in DMEM (1.8 mM Ca [Ca] and 1 mM phosphate [P]; Lonza) supplemented with fetal bovine serum (FBS) (10 %), glutamine (1 %) (Biochrom), non-essential amino acids (NEA) (1 %), penicillin and streptomycin (1 %) (Biochrom).

The A7r5 cells were seeded into 20 plates of 15 cm in diameter (152 cm^2 surface area). For Ca content analysis, 6-well plates (9.6 cm^2 surface area) were used. When cells reached 60 %-70 % confluence, the experimental conditions were added. For calcification experiments, DMEM-F12 culture medium was used to maximize the differences between non-calcifying and calcifying conditions. Non-calcifying medium (control): DMEM-F12 (1 mM Ca and P) supplemented with BSA (0.1 %), penicillin, and streptomycin (1 %). Calcifying medium: a calcifying stimulus was added to the non-calcifying medium to change the A7r5 cells phenotype to osteoblast-like; in this case, it was supplemented with Ca and P to final concentrations of 2 mM and 3 mM, respectively. In our experimental conditions, concentrations > 2 mM Ca and 3 mM phosphate induce spontaneous precipitation of $Ca_3(PO_4)$.

The 2 studies were conducted in parallel, and the culture medium was replaced every 2 days.

CALCIUM CONTENT

The degree of mineralization was assessed by Alizarin Red staining at day 6 of culture under calcifying and non-calcifying conditions. Briefly, cells were washed with phosphate-buffered saline (PBS), fixed with formaldehyde (10 % in PBS) at 4 °C for 45 minutes, and after a wash with deionized water, they were stained with 2 % Alizarin Red (pH 4.2) for 5 minutes.

PROTEOMICS STUDY

After 6 days of culture and confirming the presence of calcification, the cells were collected in 20 mL of PBS and pooled to obtain 4 samples cultured in non-calcifying medium (C1, C2, C3, and C4) and 4 samples from the culture in calcifying medium (P1, P2, P3, and P4). In both groups, the samples were solubilized by sonication in lysis buffer (7M urea, 2M thiourea, 4 % CHAPS, and 30 mM Tris). The total protein concentration was measured using the Bradford method (13) (Bio-Rad, Hercules, CA, USA).

Following the manufacturer's recommendations, 400 pmol of CyDye™ DIGE FluorDyes (GE Healthcare, Uppsala, Sweden) were added per 50 µg of protein (fluorochromes Cy3 and Cy5 were used to stain the samples and Cy2 for the internal standard, resulting from the mix of equal amounts of protein from the samples cultured under non-calcifying and calcifying conditions). The samples were stained in pairs (non-calcifying and calcifying conditions) to avoid labeling differences.

The separation of the labeled protein extracts was performed in 2D-DIGE gels (2D Fluorescence Difference Gel Electrophoresis), following the manufacturer's instructions (GE Healthcare, Uppsala, Sweden). First dimension: the samples were loaded on IPG strips (24 cm; pH 3-10 NL) (GE Healthcare). After the first dimension, the IPG strips were incubated in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6M urea, 30 % glycerol,

2 % SDS, and traces of bromophenol blue) containing 0.5 % dithiothreitol (DTT) for 15 minutes and then, in the same buffer, with 4.5 % iodoacetamide for another 15 minutes. For the second dimension, the strips were loaded onto polyacrylamide gels (12.5 %) (Ettan DALT six system, GE Healthcare, Uppsala, Sweden) and run (2W/gel) for 4 hours and 30 minutes until the bromophenol blue reached the gel's bottom. Then, the 2D gels were scanned (EttanDigerImager, GE Healthcare, Uppsala, Sweden) at 100 µm resolution with $\lambda_{ex}/\lambda_{em}$ of 488/520, 532/580, and 633/670 nm for Cy2, Cy3, and Cy5, respectively.

Three independent experiments were conducted for each condition. The image analysis was conducted with the DeCyder2D v7.0 software (GE Healthcare, Uppsala, Sweden). For spot selection, volume quantification, and normalization of the samples in the gel, the differential in-gel analysis (DIA) module was used. The biological variation analysis (BVA) module was used to compare protein spots across different gels and identify those showing significant differences.

A preparative gel in polyacrylamide (12.5 %) with 500 µg of protein (the same amount from each sample) was performed following the same procedure described above. The proteins were visualized by staining with Oriole™ Fluorescent Gel Stain (Bio-Rad, Hercules, USA); the images were acquired using a gel documentation system, ChemiDoc™ XRS+ (Bio-Rad, Hercules, USA).

Differentially expressed spots were manually excised. The digestion of the spots was performed with 12.5 ng/µL trypsin in 50 mM ammonium bicarbonate for 12 hours at 37 °C. For peptide extraction, a solution of formic acid (1 %) and acetonitrile (50 %) (Sigma Aldrich, St Louis, USA) was used.

PROTEIN IDENTIFICATION

The protein extracts were analyzed using a nanoHPLC system (Ultimate 3000, Dionex/LC Packings) with an autosampler connected to a Q-trap ion mass spectrometer (Applied Biosystems) with a nanoelectrospray ionization (ESI) source. The chromatographic column was C-18 with 75 µm diameter (Dionex/LC Packings) connected to a silica capillary to generate the electrospray. The sample injection volume used was 1 µL, and the mobile phase flow rate was 0.125 mL/min (split mode).

The mass spectrometry (MS/MS) spectra obtained were analyzed using the Analyst software and matched to the SwissProt database for protein identification using the MASCOT search engine (MatrixScience). Search parameters included a tolerance of ± 1.2 Da and possible protein changes, such as deamidation, carbamylation, and oxidation of methionine. A protein was considered identified when, at least, 2 different peptides were detected. The function and location of the identified proteins were assigned based on information from the PubMed and ExPasy databases.

Supplementary data shows a diagram of the processes performed in the proteomics study (Fig. 1) and representative images of the gels labeled with the fluorochromes Cy2, Cy3, and Cy5, and the resulting image from the combination of the three fluorochromes (Fig. 2).

STATISTICAL ANALYSIS

The analysis of the DIGE results was performed with the DeCyder2D v7.0 software (GE Healthcare, Uppsala, Sweden) using Student's t-test ($p < 0.05$). Protein spots showing expression changes above a threshold of 1.5 were selected.

RESULTS

Experimental conditions for VSMC cultivation were maintained until the day 6 of treatment, at which point an increase in Ca deposition in the cultures treated with calcifying medium was observed, as determined by Alizarin Red staining (Fig. 3).

To assess the differential protein expression in VSMCs treated with calcifying medium vs cells cultured under non-calcifying conditions, a 2D-DIGE comparative analysis was performed. Approximately a total of 1860 protein spots were detected, 121 of which were differentially expressed ($p < 0.05$), and 101 of these spots were analyzed by LC-ESI-MS/MS (Fig. 4). The obtained mass spectra were matched vs databases, and an individual score > 32 in Mascot indicated identity or homology ($p < 0.05$). A total of 20 different proteins were identified in 24 spots.

Four of all identified proteins showed increased expression in the cells cultured in calcifying medium vs those cultured in non-calcifying medium (Table I). Type I collagen and annexin A2 exhibited the largest differences between non-calcifying and calcifying conditions (ratios of 3.49 and 2.17, respectively) after 6 days.

Table II lists 16 proteins whose expression was inhibited in cells cultured in calcifying medium vs those cultured in non-calcifying medium. Tropomyosins (beta chain, alpha-1, and alpha-3) were the proteins that showed the most significant decrease when VSMCs were cultured in a calcifying medium. Additionally, some of them were identified in multiple spots, suggesting possible changes to their structure or post-translational changes. Other muscle-specific proteins that

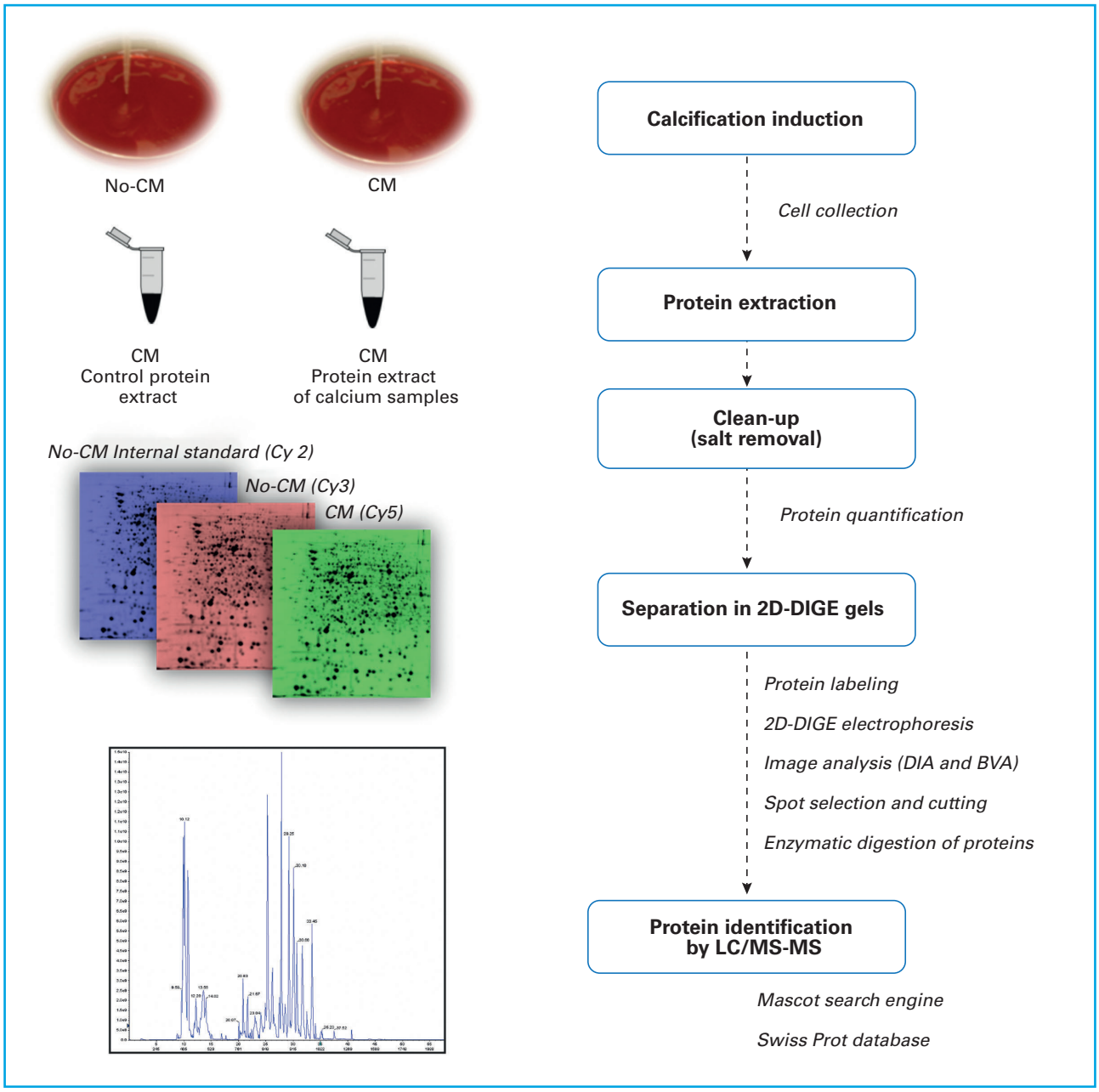

Figure 1. Sample treatment for proteomic analysis by LC-MS/MS.

reduced their expression included actin, myosin-9, desmin, alpha-actin, and other cytoskeletal proteins such as vimentin and alpha-1-A chain of tubulin.

Disulfide isomerase proteins A6 and A1 and endoplasmin were 3 chaperones localized in the endoplasmic reticulum that reduced their expression more than 6, 3, and 2 times, respectively, under calcifying vs non-calcifying conditions. A decrease in the expression of nucleobindin-1, a Ca-binding protein localized in the Golgi apparatus, and P3H1, which has a structural function and is a component of the extracellular matrix, was also observed.

DISCUSSION

The publication of numerous studies on VC and CKD has partially contributed to understanding the process of CKD-related VC. In this regard, omics techniques (genomics, transcriptomics, and proteomics, along with other analysis techniques) provide a powerful tool to obtain and integrate biological information about the VC process (14). Proteins—the main effectors of most biological processes—are the most suitable molecules to be used as biomarkers or targets for the treatment of diseases due to the close relationship between proteins and phenotypes. For this reason, results obtained

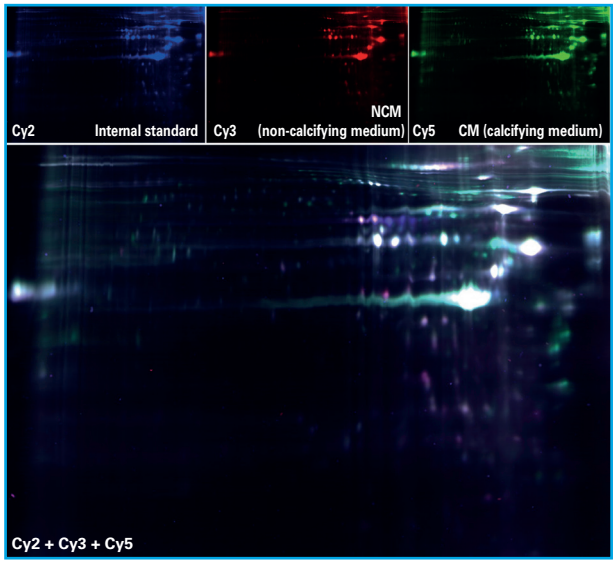

Figure 2. Representative images of the gels resulting from the electrophoresis of proteins from cells cultured in non-calcifying (Cy3) and calcifying medium (Cy5), the internal standard (Cy2), and the resulting combination of the 3 (Cy2 + Cy3 + Cy5).

in proteomic studies could be more reproducible than those in genomics and transcriptomics (14).

After mass spectrometry analysis of the protein extracts obtained from VSMCs cultured for 6 days in calcifying and non-calcifying media, 20 proteins were identified with high reliability.

Of the 4 proteins identified that showed increased expression in calcifying medium, the one with the greatest change was the alpha chain of type I collagen. Several studies relate the increase in collagen I

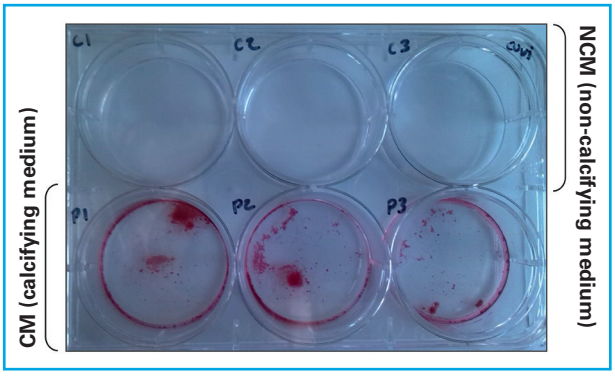

Figure 3. Alizarin red staining of CMLV after 6 days of culture in non-calcifying and calcifying conditions.

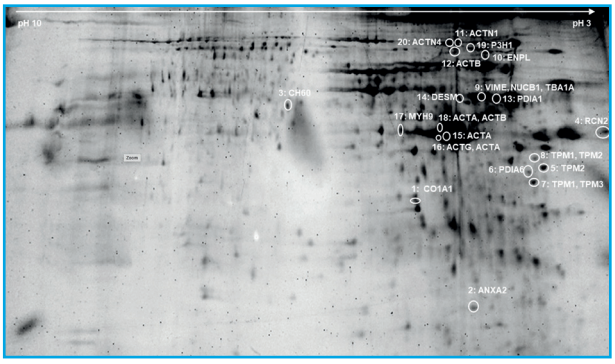

Figure 4. Map of the differential protein expression profile in VSMC cultures under calcifying vs non-calcifying conditions. Preparative gel. The spot number and the MASCOT symbol of the identified proteins are indicated.

levels to VC. Collagen I and III are the main components of the extracellular matrix whose levels remain relatively stable under physiological conditions.

Table I. Overexpressed proteins in CMVL A7r5 cells cultured under calcifying conditions vs those cultured under non-calcifying conditions, identified by LC-MS/MS

Spot No.	Mascot symbol	Protein name	Function	Localization	*p-value*	Fold change ratio	Mascot score	Mass (Da)	IP	Peptides
1	CO1A1_RAT	Alpha-1 collagen chain (I)	Structural	Extracellular matrix	0.0023	3.49	80	137869	9.28	2 (1)
2	ANXA2_RAT	Annexin A2	Ca binding and interaction with cytoskeleton	Extracellular matrix	0.018	2.17	111	38654	7.53	3 (3)
3	CH60_RAT	Mitochondrial 60 kDa heat shock protein	Cellular stress (chaperone)	Mitochondrial matrix	0.0072	1.57	136	60917	5.91	2 (2)
4	RCN2_RAT	Reticulocalbin-2	Ca binding	RER	0.017	1.54	131	37410	4.23	3 (2)

RER: rough endoplasmic reticulum. The spot number, Mascot symbol, protein name, function, localization, theoretical mass (Da), isoelectric point (IP) obtained from the databases (ie, without considering possible post-translational alterations or changes), as well as other mass spectrometry analysis data, such as the number of peptides identified (with the number of unique peptides indicated in parentheses), are shown.

Table II. Proteins inhibited in CMVL A7r5 cell cultures subjected to calcification treatment vs control

Spot No.	Mascot symbol	Protein name	Function	Localization	*p-value*	Fold change ratio	Mascot score	Mass (Da)	IP	Peptides
5	TPM2_RAT	Beta tropomyosin	Cytoskeleton (structural)	Cytoplasm	0,0075	-10,4	170	32817	4,66	6 (5)
6	PDIA6_RAT	Disulfide isomerase A6 protein	Chaperone	RER	0,016	-6,45	83	48100	4,95	2 (1)
7	TPM1_RAT	Alpha-1 tropomyosin chain	Cytoskeleton (structural) and cell adhesion	Cytoplasm	$6\ e^{-006}$	-6,39	299	32661	4,69	11 (9)
	TPM3_RAT	Alpha-3 tropomyosin chain	Cytoskeleton (structural)	Cytoplasm			100	28989	4,75	5 (3)
8	TPM1_RAT	Alpha-1 tropomyosin chain	Cytoskeleton (structural) and cell adhesion	Cytoplasm	0,0055	-6,21	89	32661	4,69	2 (2)
	TPM2_RAT	Beta tropomyosin	Cytoskeleton (structural)				89	32817	4,66	2 (2)
9	VIME_RAT	Vimentin	Cytoskeleton	Cytoplasm	0,018	-5,17	248	53700	5,05	7 (7)
	NUCB1_RAT	Nucleobindin-1	Ca binding	Golgi apparatus			138	53474	5,01	4 (3)
	TBA1A_RAT	Alpha-1 tubulin	Cytoskeleton	Cytoplasm			132	50104	4,94	2 (2)
10	ENPL_RAT	Endoplasmin	Chaperone (oxidative stress), Ca binding	RER	0,02	-3,49	235	92713	4,69	4 (4)
11	ACTN1_RAT	Alpha-actinin-1	Cytoskeleton and internal cell motility	Cytoplasm	0,0098	-2,63	523	102896	5,23	16 (13)
12	ACTB_RAT	Actin (cytoplasmic) 1	Cytoskeleton (structural)	Cytoplasm	0,0098	-2,63	53	41710	5,21	2 (1)
13	PDIA1_RAT	Disulfide isomerase A1 protein	Chaperone (stress)	RER	0,016	-2,37	278	56916	4,77	5 (5)
14	DESM_RAT	Desmin	Structural	Cytoplasm	0,0043	-2,32	47	53424	5,21	2 (1)
15	ACTA_RAT	Smooth muscle actin (aorta)	Cytoskeleton and cell motility	Cytoplasm	0.032	-2,3	159	41982	5,24	7 (5)
16	ACTG_RAT	Actin (cytoplasmic) 2	Cytoskeleton and cell motility	Cytoplasm	0,032	-2,3	317	41766	5,31	7 (5)
	ACTA_RAT	Smooth muscle actin (aorta)					145	41982	5,24	7 (3)
17	MYH9_RAT	Myosin-9	Cytoskeleton	Cytoplasm	0,017	-2,15	168	226197	5,49	5 (3)
18	ACTA_RAT	Smooth muscle actin (aorta)	Cytoskeleton and cell motility	Cytoplasm	0,015	-1,93	127	41982	5,24	4 (3)
	ACTB_RAT	Beta-actin (cytoplasmic) 1					354	47710	5,24	9 (8)
19	P3H1_RAT	Prolyl 3-hydroxylase 1	Structural, Extracellular matrix component	RER secreted to the extracellular matrix	0,05	-1,88	224	82338	5	6 (6)
20	ACTN4_RAT	Alpha-actinin-4	Cytoskeleton and internal cell motility	Cytoplasm	0,049	-1,5	326	104849	5,27	6 (6)

RER: rough endoplasmic reticulum.

However, elevated phosphate and Ca levels increase collagen I expression, favoring the differentiation of VSMCs into osteoblast-like cells (15). Another protein involved in collagen biosynthesis and maturation is prolyl-3-hydroxylase (P3H1), a proteoglycan with enzymatic activity necessary for the proper formation of collagen helices, whose expression was reduced under calcifying conditions, consistent with other studies analyzing protein expression profiles in VSMCs under calcifying conditions (16). Furthermore, mice deficient in this protein showed hypermineralization of the bone matrix (17). Changes in the characteristics of the extracellular matrix and behavior among its constituents influence not only the mechanical properties of connective tissues but also contribute to modulating the cellular phenotype, altering protein expression, cytoskeleton organization, and, consequently, intracellular signaling pathways.

Other proteins from the rough endoplasmic reticulum (RER) whose expression was altered include disulfide isomerase proteins A1 and A6 (PDIA1, PDIA6), involved in disulfide bond formation, isomerization, and reduction, acting alongside chaperones in the regulation of misfolded proteins. The expression of these proteins was found to be inhibited under calcifying conditions, and although there is no evidence of PDIA6 role in VC, changes in PDIA1 expression have been associated with increased VC and aortic calcification (18,19).

Among this group of proteins involved in folding, which showed an impaired expression in this study, is the 60 kDa mitochondrial heat shock protein (HSP60) and endoplasmin (ENPL). The expression of HSP60 in vascular disease has been correlated with the severity of atherosclerosis and the anti-inflammatory response (20). Several studies have demonstrated that HSP expression is closely related to the VC process (21-23). On the other hand, although no association between ENPL and VC has been described, this protein participates in the normal differentiation of cardiac, smooth, and skeletal muscle cells (24), so changes in its expression could alter their normal differentiation.

In the present study, an increase in Ca^{2+}-binding proteins, such as reticulocalbin-2 (Rcn-2) and annexin A2, was also observed. Ca-binding proteins (CaBP) participate actively in many cellular processes through specific domains, such as Ca^{2+} homeostasis or signaling pathways (25). Mineralization initiation requires the entry of Ca^{2+} into matrix vesicles. Specifically, Rcn-2 has been described as a mediator of VC, increasing the expression of osteogenic markers and reducing the expression of contractile phenotype markers in VSMCs (26). On the other hand, annexins are a family of Ca-dependent membrane-binding proteins. Different studies have shown the importance of annexins in matrix vesicle formation through Ca entry and the initiation of mineralization (27) and the similarities between the process of matrix vesicle formation from VSMC membranes and chondrocytes, including the enrichment of annexins A2, A5, and A6 as triggers for the mineralization process (28). Another Ca-binding protein, but one that was inhibited in this study, is nucleobindin 1 (NUCB1), localized in the Golgi apparatus, where it plays an important role in Ca homeostasis (29), although no studies have yet associated it with VC.

Additionally, in line with previous work conducted in in vivo and in vitro VC models and proteomic studies, the results of this work showed a decrease in the expression of structural proteins and the deregulation of cytoskeletal proteins in VSMCs (16,30,31). Among this group of proteins, those that experienced the greatest decrease were typical muscle proteins (tropomyosin, actin, desmin, myosin, etc.). This may be interpreted as a loss of the muscle phenotype of VSMCs as a result of exposure to high concentrations of phosphate and Ca, as previously described in other studies (32).

One of the issues with VC is that it is diagnosed at late stages, once it is already established, and no therapies specifically targeting VC have been described to date. Furthermore, the diagnostic techniques used, mainly based on imaging techniques, may be limited due to factors such as cost, availability, and radiation exposure (33). Therefore, the identification of minimally invasive, sensitive, and specific circulating biomarkers that could identify the presence of VC in early stages would present a highly interesting alternative. The proteins identified in this study could help in the search for biomarkers to identify patients at higher risk of developing VC or could even be used as therapeutic targets once validated in other studies.

This type of study presents several limitations, as protein identification is limited by the quantity and quality (low concentration of salts, nucleic acids, lipids, etc.) of the obtained protein extract, which directly depends on the effectiveness of the extraction method, and the sample preparation, which is crucial for the quality of the results (34). In this study, in addition to the proteins identified with high reliability, others with low scores were also detected, which could be due to various reasons (35): in the cut spots, the concentration of some proteins may be much lower than that of others, and post-transcriptional changes or proteolysis of some proteins could alter the availability of peptides for identification. Of note, being an in vitro calcification model, it does not allow for a direct comparison between the CKD state and a healthy control, as CKD involves systemic factors and physiological conditions that are not fully captured in this model. Another limitation to consider is that the cell line used was from rats, so the translation of the results to humans is limited. On the other hand, the characterization of the protein profile of the cells was carried out in a calcification model that was maintained for 6 days. Analyses at other points in the process would contribute to a deeper understanding of vascular calcification. It is possible that in models where calcification could be

maintained for longer periods, the levels of some proteins could have been higher.

CONCLUSIONS

The results obtained confirmed a change in the phenotypic expression of muscle cells, with a decrease in typical muscle and cytoskeletal proteins. Moreover, it was possible to identify changes in the expression of proteins previously not linked to VC, which could participate in this process. Considering the results obtained in this study, it would be very useful to conduct complementary experiments to clarify the role of these and other differentially expressed proteins in the VC process. These proteins could be used as markers of the early stages of the disease or as target molecules for the development of new therapeutic strategies that could reduce the morbidity and mortality of patients with CKD.

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Revista de Osteoporosis y Metabolismo Mineral

Special Article

Dialogues between basic and clinical researchers: hyperphosphatemia

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Abstract

Physiological regulation of mineral metabolism is determined by serum levels of phosphorus, FGF23, Klotho, PTH, calcidiol, and calcium. When renal function is normal, the kidney, bone, parathyroid tissue, and intestine collaborate effectively in maintaining this regulation, even in cases of phosphorus excess.

The issue arises when renal function is compromised, as the regulators of mineral metabolism become altered, including decreased soluble Klotho, increased PTH and FGF23, and subsequent reductions in calcidiol and calcium. These changes lead to vascular and bone abnormalities, with significant impacts on the morbidity and mortality of renal patients.

From a therapeutic standpoint, dietary phosphorus restriction is the initial approach for controlling hyperphosphatemia in patients with chronic kidney disease (CKD). When dietary measures are insufficient, phosphorus binders are used to limit intestinal absorption of this ion.

In summary, it is crucial to identify and treat hyperphosphatemia adequately to achieve comprehensive clinical improvements, including a significant reduction in mortality.

Keywords:
Phosphorus. Renal disease. Morbidity and mortality.

PATHOPHYSIOLOGICAL AND MOLECULAR SIGNIFICANCE OF HYPERPHOSPHATEMIA

The physiological regulation of mineral metabolism is governed by several factors contributing to its maintenance and regulation to varying degrees. For hyperphosphatemia, the critical determinant is serum phosphorus levels, though FGF23, Klotho, PTH, calcidiol, and calcium also play vital roles. Under normal renal function, the organism effectively manages mineral metabolism through the coordinated activity of the kidney, bone, parathyroid tissue, and intestine. However, compromised renal function impairs phosphorus excretion, leading to its accumulation. As kidney disease progresses, the levels of various mineral metabolism regulators become increasingly disturbed. While some controversies remain, it appears that the initial event is a decrease in soluble Klotho levels. Additionally, excess phosphorus leads to increased PTH and

Received: 16/09/2024 • Accepted: 26/09/2024

Acknowledgments: this study was funded by Instituto de Salud Carlos III (ISCIII)-Health Research Fund (PI16/00637, PI17/00715, PI19/00532, PI20/00753, and PI22/00195), the European Regional Development Fund (FEDER), the Science, Technology, and Innovation Plan 2013-2017 and 2018-2022 of the Principality of Asturias (IDI-2018-000152, IDI-2021-000080, IDE/2024/000706), the Íñigo Álvarez de Toledo Renal Foundation (FRIAT), the ISCIII REDinREN network (RD16/0009/0017 and RD16/0009/0018), and RICORS2040 (Kidney Disease; RD21/0005/0019).

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

Artificial Intelligence: the authors declare that they did not use artificial intelligence (AI) or any AI-based tools for the drafting of this article.

Rodríguez García M, Naves Díaz M. Dialogues between basic and clinical researchers: hyperphosphatemia. Rev Osteoporos Metab Miner 2024;16(4):149-153

DOI: 10.20960/RevOsteoporosMetabMiner.00062

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FGF23 levels, followed by reductions in calcidiol and calcium (1-3). These changes culminate in vascular and bone abnormalities with profound effects on the morbidity and mortality of renal patients.

Although clinical and experimental settings make it challenging to isolate the role of each factor due to tissue interconnections, animal models have clarified the contributions and specific impacts of these elements.

One of the main regulators of phosphorus is FGF23, primarily expressed in bone tissue and synthesized by osteocytes (4). Osteocytes detect excess phosphate and stimulate FGF23 synthesis and secretion via receptor FGFR1(5) and phosphate transporter Pit2 (6). FGF23 acts on renal tubules with Klotho to internalize and degrade the NaPi2a phosphorus transporter, decreasing reabsorption and enhancing excretion.

FGF23 also acts independently on the heart, promoting cardiac damage via FGFR4 through the calcineurin pathway (7,8), although it does not have similar deleterious vascular effects (9).

The deficiency or absence of Klotho in experimental models has demonstrated an increase in cardiac hypertrophy and fibrosis, both at the molecular and histological levels (10,11). Similarly, the absence of Klotho produces an accelerated aging phenotype at the vascular and bone levels (12). Klotho deficiency has also shown vascular and bone alterations (3,13). In contrast, the addition of soluble Klotho in experimental models is capable of preventing or reversing cardiac damage (10).

Experimental models of parathyroidectomy with hyperphosphatemia, maintaining PTH levels within normal ranges through exogenous administration, have demonstrated that phosphorus alone van cause vascular and bone damage. However, this effect is much more pronounced when PTH is very elevated (14), leading to bone deterioration at the trabecular bone level, but especially at the cortical bone level (15). Additionally, there is an increase in calcium content in the arteries with an exacerbated rise in the expression of osteogenic genes, such as Runx2 or osterix, along with sharp decreases in the expression of vascular phenotype genes, such as alpha-actin (15). Increases in PTH have also been associated with cardiac alterations, where an increase in hypertrophy and particularly in cardiac fibrosis has been observed at both the histological and molecular levels (elevations in the gene expression of collagen I, TGF beta, or fibronectin) (16).

Experimental and clinical studies have shown that vitamin D or calcidiol deficiency is associated with an increased risk of cardiovascular disorders (17,18). However, to date, the bone is the only organ where vitamin D has demonstrated causality, proving to be an effective treatment for reducing the risk of hip fractures and non-vertebral fractures (19).

The role of calcium is less clear. Probably, the more refined management of the organism to maintain calcium homeostasis makes the effects at the systemic level much more subtle. In the presence of chronic kidney disease (CKD) and hyperphosphatemia, decreases in calcium could have a more potent effect than increases, mainly at the cardiovascular level (20), probably partly due to the direct effect that hypocalcemia exerts on the parathyroid gland, stimulating the synthesis and secretion of PTH (21).

In vitro studies have shed more light on the systemic effects of hyperphosphatemia and the pathophysiological mechanisms involved. At vascular level, experimental studies are confirmed: the additive effect of phosphorus increases, sufficient available calcium, but especially PTH, on vascular damage, as they promote vascular calcification through the increase in the expression of typically osteogenic genes (Runx2, osterix, alkaline phosphatase) and the drastic decrease in genes related to the vascular and contractile phenotype, such as alpha-actin (15). At this level, the role of soluble Klotho is fundamental for maintaining vascular health, as its addition to in vitro models in vascular smooth muscle cells exposed to calcifying stimuli prevents the increase in the expression of typically osteogenic genes, reduces extracellular calcium deposition, and prevents the loss of alpha-actin, the main protein for maintaining the vascular phenotype (3). There are still doubts about the mechanism through which this protective effect could be exerted, but there is some data suggesting that one of these mechanisms might be the increase in autophagic flow, which would prevent the vascular calcification process (3), although more studies are needed to demonstrate this causality.

Recently, our group has shown that this loss of vascular phenotype with a predisposition to vascular calcification is mainly due to the loss of microRNA 145, the predominant microRNA in the vascular wall responsible for maintaining its contractile phenotype (22). These results, initially revealed in nephrectomized animals with hyperphosphatemia, but also in animals with normal renal function and hyperphosphatemia, have been confirmed in vitro in vascular smooth muscle cells subjected to calcifying stimuli from excess phosphorus, calcium, and/or PTH (23). These results could have important clinical implications, as in the general population, the expression of this microRNA shows a ROC curve with an area under the curve of 0.83 (22), showing that microRNA 145 has a high predictive power for vascular calcification. These novel findings suggest that the combination of non-invasive, low-cost, and simple biomarkers would constitute an important predictor of vascular damage in not only the renal population but also the general population.

The regulation of microRNA 145 levels by vitamin D is another revealing aspect, as vitamin D increases the expression of microRNA 145 (24). Therefore, maintaining calcidiol levels could positively impact vascular health through the maintenance of microRNA 145 levels (25), as confirmed by other clinical studies in both renal and general populations.

DETECTION, SIGNIFICANCE, AND THERAPEUTIC MANAGEMENT

Hyperphosphatemia originates when the phosphorus entering the extracellular fluid exceeds the amount that can be excreted. One of the most common causes of reduced phosphorus excretion is acute and chronic kidney disease. Other causes include the mobilization of intracellular phosphorus to the extracellular fluid (lactic acidosis, diabetic ketoacidosis, or severe hyperglycemia), acute phosphorus overload (endogenous or exogenous, such as tumor lysis syndrome, muscle necrosis, or excessive laxative intake), or increased tubular phosphate reabsorption (hypoparathyroidism, acromegaly, fibroblast growth factor receptor inhibitors, vitamin D, or tumoral calcinosis).

Hyperphosphatemia has been linked to endothelial dysfunction, arteriosclerosis, and calcification of the arterial media, both generalized and coronary, as well as cardiac valve calcification, myocardial fibrosis leading to ventricular wall stiffness, diastolic dysfunction, heart failure, and arrhythmias (26).

There is extensive scientific evidence linking elevated serum phosphorus with increased cardiovascular events and mortality, both in the general population (27) and in those with chronic kidney disease (20,28), and especially among dialysis patients (a population at maximum risk for developing hyperphosphatemia) (29,30). Hyperphosphatemia is currently considered a non-traditional cardiovascular risk factor.

In hemodialysis patients, a U-shaped relationship has been described between serum phosphorus levels and mortality, where both below and above the recommended range are associated with increased mortality risk. Moreover, improved control of serum phosphorus in patients with elevated baseline levels is significantly associated with better survival during a 3-year follow-up period (30).

It should be considered that serum phosphorus levels can fluctuate throughout the day, with postprandial increases. For this reason, fasting serum phosphorus has been most closely associated with higher cardiovascular mortality risk, both in the general population and in patients with chronic kidney disease (31).

On the other hand, high-phosphorus diets have been linked to increased blood pressure, both through previously mentioned mechanisms (arteriosclerosis, arterial wall stiffness), and due to their effect on increased sodium reabsorption at the renal tubular level and activation of the sympathetic nervous system (26).

Hyperphosphatemia has also been associated with the development of CKD in healthy individuals, as well as with disease progression in patients who already have it (32).

Additionally, a recent study has linked serum phosphorus values in hemodialysis patients with an increased risk of fragility fractures, suggesting that serum phosphorus could be a new risk marker for bone fractures (33).

Therapeutic approach varies depending on whether the hyperphosphatemia is acute or chronic. In cases of acute hyperphosphatemia, resolution can occur within 6 to 12 hours if renal function remains intact. Phosphorus excretion can be increased with saline infusion, although this could lower serum calcium concentration due to dilution, requiring caution, especially in the presence of severe hypocalcemia, due to the associated life-threatening risk. In cases of severe symptomatic hypocalcemia and renal function deterioration, hemodialysis may be necessary for effective management.

For patients with CKD, the initial measure for controlling hyperphosphatemia is to restrict dietary phosphorus intake (34), which is classified into two types: organic, found mainly in protein-rich foods, and inorganic, present in additives, carbonated beverages, and processed foods. Inorganic phosphorus is less biologically relevant but has a considerably higher absorption rate.

It is very important to control both phosphorus levels and secondary hyperparathyroidism in these patients, due to all the previously mentioned implications.

Phosphate binders work by limiting intestinal absorption of this ion (34). They are classified based on their calcium content, into calcium-containing binders such as calcium carbonate or calcium acetate, and calcium-free binders like sevelamer carbonate, lanthanum carbonate, and sucroferric oxyhydroxide, which must be taken with meals to be effective.

Therefore, it is crucial to emphasize the importance of identifying and appropriately treating hyperphosphatemia to achieve an overall improvement in clinical outcomes, including a significant reduction in mortality.

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