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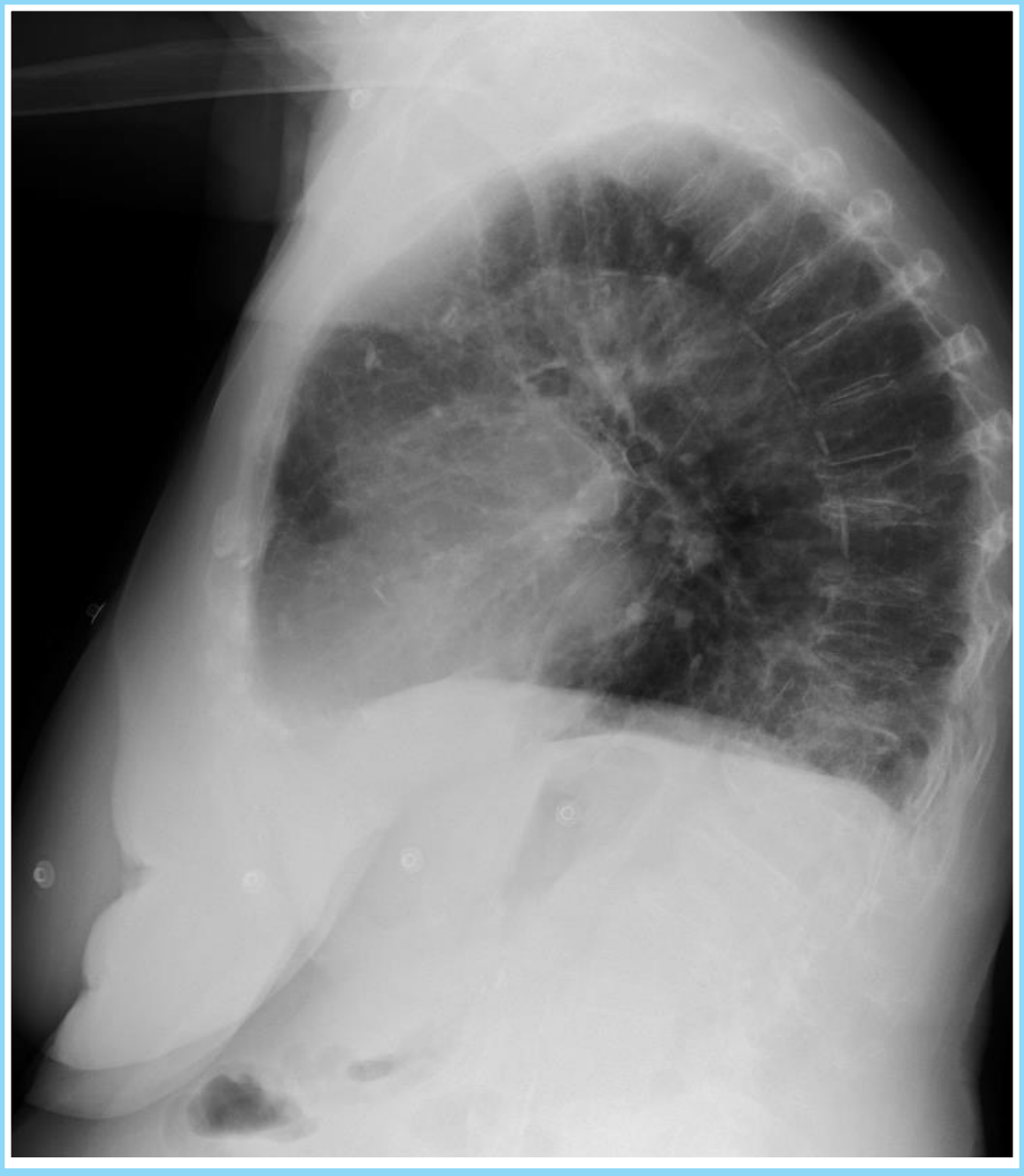

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Summary

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Originals

Review

Cover image:

Lateral chest X-ray – A diagnostic opportunity

Department of Internal Medicine. Hospital Universitario Marqués de Valdecilla. Santander, Spain

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Original

Study of bone turnover biomarker behavior within the first year post-kidney transplant

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Abstract

Introduction: kidney transplantation (KT) poses a risk for decreased bone strength, especially during the first-year post-kidney transplant when the dose of corticosteroids used is higher. The objective of the study is to analyze the behavior of bone formation and resorption biomarkers during the first year post-KT.

Material and methods: observational, prospective, and single-center study including 123 patients admitted for KT. Routine parameters related to mineral metabolism and, in a subgroup of patients, bone formation biomarkers (bone alkaline phosphatase [BALP] and procollagen type 1 N-terminal propeptide [P1NP]) and resorption biomarker (tartrate-resistant acid phosphatase 5b [TRAP5b]) were determined peri-transplant, at 6 and 12 months.

Results: parathyroid hormone (PTH) decreased significantly and markedly during the first semester (239 ± 124 vs 91 ± 40 ng/L), remaining stable during the second semester (92 ± 40 ng/L). An increase in BALP (9.03 ± 3.95 vs 11.18 ± 4.71 µg/L; $p < 0.001$) and P1NP (48.4 ± 35.7 vs 64.6 ± 42.6 µg/L; $p < 0.001$) was observed at 12 months post-KT. No significant changes were observed in TRAP 5b. Patients who received anti-resorptive treatment compared to untreated patients showed significantly lower levels of BALP (9.39 vs 11.87 µg/L), P1NP (37.6 vs 70.3 µg/L), and TRAP5b (2.59 vs 4 U/L) at 12 months.

Conclusion: during the first year of KT, there is an increase in bone formation biomarkers, despite a decrease in PTH levels. Bone turnover biomarker levels at one-year post-KT are lower in patients treated with anti-resorptive agents.

Keywords:
Bone turnover markers. Kidney transplantation. Anti-resorptive treatment.

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INTRODUCTION

Kidney transplantation (KT) poses an additional risk for bone disease and osteoporosis, which will lead to an increase in morbidity and mortality. A 7-10 % of kidney transplant recipients will suffer at least one fracture throughout their lives (1,2). The post-KT bone phenotype is established by the combination of several factors: a) mineral metabolism disorders during the advanced chronic kidney disease (CKD) phase that may persist post-KT, especially hyperparathyroidism; b) the effects of immunosuppressants, mainly glucocorticoids; and c) traditional risk factors for osteoporosis such as nutritional status, hormone-dependent or aging-related phenomena (3,4). The latest update of the nephrological guidelines on the CKD-MBD (chronic kidney disease-mineral and bone disorder) complex suggests the assessment of fracture risk at any stage of CKD G1-5T through the measurement of bone mineral density (BMD) (4,5). DEXA (dual-emission X-ray absorptiometry) is the standard method for assessing fracture risk not only in the general population but also in CKD (6,7); however, it has some limitations. The first drawback is that it only provides information on bone quantity, without evaluating its quality, which represents a particularly significant limitation in the context of secondary osteoporosis due to metabolic diseases. In CKD, various factors, such as the uremic environment, prolonged use of corticosteroids, and comorbidities such as diabetes mellitus, along with renal osteodystrophy marked by abnormalities in bone turnover, will considerably affect bone quality. The second important limitation of DEXA is that it does not allow early detection of changes in bone after the start of anti-fracture treatment. In this regard, bone turnover markers (BTMs) could mitigate these limitations. Although BTMs are not useful for the diagnosis of osteoporosis, they could complement DEXA in identifying patients at high risk of fracture. The SEIOMM guidelines (8) especially recommend their use (evidence B) to evaluate the response to anti-osteoporotic treatment early (evidence 2a) and to monitor adherence to it, as they can be determined iteratively without irradiating the patient.

Biomarkers are divided into circulating factors that affect bone turnover (e.g., parathyroid hormone [PTH]) and bone turnover markers that reflect the number and/or activity of bone cells. The latter are divided into 2 categories: formation markers [bone alkaline phosphatase (BALP), procollagen type 1 N-terminal propeptide (P1NP)] and bone resorption marker [tartrate-resistant acid phosphatase 5b (TRAP5b)]. BTMs would not only allow for faster and more dynamic improvement in monitoring and therapeutic efficacy but can also provide information on the type of renal osteodystrophy. Bone turnover biomarkers have a high negative predictive value in relation to bone turnover in CKD, so they will be useful to rule out the presence of high or low bone turnover (9). The KDIGO (Kidney Disease: Improving Global Outcomes) guidelines suggest measuring PTH or BALP, as extreme values could indicate bone turnover (5). Experience on the behavior of other BTMs, such as P1NP or TRAP5b in renal patients, is limited, so new evidence on their behavior is needed to recommend more liberal use. The use of BTMs in the future is promising, as it could help to better stratify fracture risk in renal patients, individualize treatment, and monitor therapeutic response earlier. The objective of this study is to analyze the behavior of bone formation and resorption biomarkers during the first year post-KT, as well as the influence of anti-resorptive treatment on the evolution of biomarkers.

MATERIAL AND METHODS

STUDY DESIGN AND POPULATION

This is a substudy of the "Study of bone strength measured *in vivo* by impact microindentation in post-kidney transplant recipients" in a cohort of patients older than 18 years, admitted for KT from living or deceased donors at the Fundació Puigvert between May 2019 and August 2022. It is a prospective, observational, and single-center study where patients were assessed peri-transplant, at 6 months, and 1 year post-KT. The study was approved by the CEIm (Ethics Committee for Research with Medicinal Products) of Fundació Puigvert. Adult patients admitted for KT were included. Exclusion criteria were history of recent treatment (< 1 year) with denosumab, bisphosphonates, or teriparatide; refusal to sign informed consent.

VARIABLES

Demographic data, comorbidities, and treatment-related data were collected from patient medical records. In addition, a biomarker analysis of bone formation (BALP, P1NP) and resorption (TRAP 5b) was performed in a subgroup of patients.

IMMUNOSUPPRESSION AND MINERAL METABOLISM PROTOCOL

Patients were treated with an individualized immunosuppression regimen with corticosteroids, calcineurin inhibitor, mycophenolate, and polyclonal lymphocyte serum (thymoglobulin) according to the center's clinical protocol. The corticosteroid

dose was: 200 mg of intravenous methylprednisolone on the day of KT, 1 mg/kg/day of oral prednisone at 24 hours, and progressive reduction over the next 3 months to a maintenance dose of 5 mg/day. Treatment for mineral metabolism parameters was discontinued in the immediate post-KT period and restarted later at the discretion of the treating physician. According to protocol, based on clinical practice guidelines (8,10), the initiation of anti-resorptive treatment with denosumab 60 µcg/semi-annually or oral bisphosphonates (usually alendronic acid 70 mg/weekly) was recommended or not. Initiation of treatment was recommended in postmenopausal women and men > 40 years with a T-score < -1.5 SD or a history of fragility fracture. In premenopausal women and men < 40 years, treatment was suggested when the Z-score < -3 SD or they had experienced a fragility fracture.

ETHICAL CONSIDERATIONS

To conduct this study, a protocol was created and approved by the Ethics Committee for Research with Medicinal Products (CEIm Fundació Puigvert IUNA) on June 5th, 2020, with reference number C2019/21). All participants signed an informed consent form to participate in the study. A specific patient information sheet was developed for study participants. Both documents have been prepared for evaluation and approval by the Competent Authorities. All data have been processed in accordance with Regulation (EU) 2016/679 of the European Parliament and of the Council of April 27, 2016, on Data Protection (GDPR), and the corresponding Spanish Organic Law 3/2018, of December 5, on the protection of personal data and guarantee of digital rights. The researchers committed to conducting this study in accordance with the guidelines of Good Clinical Practice and the Declaration of Helsinki.

BIOCHEMICAL ANALYSIS

Blood samples were obtained in the morning under fasting conditions between 8:00 and 10:00 a.m. Serum concentrations of calcium, phosphate, magnesium, PTH, alkaline phosphatase, and calcidiol (25-OH-vitamin D) were measured according to routine clinical practice on a Cobas 6000 analyzer (Roche Diagnostics) within a maximum of 3 months pre-KT. The immediate post-KT determination of BTMs (bone ALP, P1NP, and TRAP5b) was performed at 2.2 ± 1.83 days post-KT. The sample was centrifuged at 3000 rpm for 10 minutes, aliquoted, and the aliquots were frozen at -80 °C until processing.

P1NP was measured using an automated electrochemiluminescence immunoassay (ECLIA) on the Cobas 6000 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The inter-assay coefficient of variation was 2.6 and 1.9 % for concentrations of 30 and 158 ng/mL, respectively. BALP was analyzed by automated chemiluminescence immunoassay with paramagnetic particles (Access Ostase®) on a Beckman-Coulter Access analyzer (Beckman_Coulter; Brea, CA, USA) with an inter-assay coefficient of variation of 4.8 and 5.3 % for concentrations of 10 and 47 µg/L, respectively. Finally, TRAP-5b was analyzed using a kinetic method based on the hydrolysis of the substrate α-naphthyl phosphate to α-naphthol, with Spinreact reagents, adapted to a Cobas Mira Plus analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The inter-assay coefficient of variation was 5.8 and 3.9 % for concentrations of 29 and 63 U/L, respectively.

STATISTICAL ANALYSIS

Qualitative variables were expressed as absolute frequencies and percentages. Quantitative variables were expressed as mean, standard deviation (SD), median, and quartiles. The Kolmogorov-Smirnov test was used to assess the normality of the distributions. The Wilcoxon test was used to analyze the evolution of biomarker levels in the entire cohort of patients. The Mann-Whitney U test was used to compare results based on treatment. The characteristics of treated and untreated patients were compared using the Chi-square test (or Fisher's exact test for frequencies < 5) for the comparison of categorical variables or the Mann-Whitney U test for quantitative variables.

Finally, multivariate linear regression models were developed to evaluate changes in BALP, P1NP, and TRAP 5b (12 months vs immediate post-KT), adjusting for the immediate post-KT value of the biomarker, age, sex, diabetes mellitus, renal function, corticosteroid dose, and PTH. Results were expressed as beta coefficient (β), 95 % confidence intervals, and *p*-value. For all tests, *p*-values < 0.05 were considered statistically significant. The R Studio statistical package (version 2.5.1) was used for the analyses.

RESULTS

BASELINE CLINICAL CHARACTERISTICS AND MINERAL METABOLISM TREATMENT

The patients' mean age was 55 ± 11 years, 69.9 % were men, and the mean body mass index (BMI) was 25.8 ±

3.8 kg/m^2. At the time of transplantation, 52.8 % and 15.4 % were on chronic hemodialysis and peritoneal dialysis programs, respectively, and 30.9 % were in the pre-dialysis stage. The mean time on pre-transplant dialysis was 24 ± 15 months. The causes of CKD were: 20.5 % glomerular, 9.8 % urological, 19.7 % polycystic kidney disease, 9 % chronic interstitial nephropathy, 8.2 % diabetic nephropathy, 4.1 % of vascular origin, and 28.7 % of undetermined origin. 22.8 % of the patients were diabetic, and 10.8 % had a history of fracture. The mean follow-up time was 12.5 ± 2.9 months. The cumulative dose of corticosteroids was 2.7 ± 0.3 g 1 post-KT. A total of 15 (13.4 %) patients experienced graft rejection during the first year. All patients were discharged after KT with oral calcium supplements and vitamin D supplements, with treatment maintained at 12 months in 66.4 % (mean dose: 433 ± 346 mg/day) and 75 % (mean dose: 798 ± 588 IU/day), respectively.

MINERAL METABOLISM LABORATORY PARAMETERS

Table I shows the mineral metabolism parameters prior to KT, at 6 and 12 months. Overall, the changes were significant, especially during the first 6 months, stabilizing during the second semester. PTH decreased significantly and markedly within the first semester (239 ± 124 vs 91 ± 40 ng/L; $p < 0.0001$), remaining stable within the second observation period (92 ± 40 ng/L). Serum phosphate levels decreased significantly at 12 months post-KT (1.75 ± 0.5 vs 1.13 ± 0.22 mmol/L; $p < 0.0001$), while calcium levels increased (2.25 ± 0.18 vs 2.4 ± 0.11 mmol/L; $p < 0.0001$) within the first year. Magnesium, however, decreased from 1.03 ± 0.16 vs 0.72 ± 0.11 mmol/L. Calcidol levels increased significantly during the first year of KT (19.3 ± 10 vs 29.3 ± 9 ng/mL).

BONE TURNOVER BIOMARKERS

The evolution of bone formation and resorption biomarkers is shown in table II and figure 1. The values of P1NP, BALP, and TRAP5b determined in the immediate post-KT period were 48.4 ± 35.7 µg/L, 9.03 ± 3.95 µg/L, and 4.25 ± 2.52 U/L, respectively. The estimated mean dose of prednisone received at that time was 303 ± 150 mg. P1NP levels were inversely correlated with the cumulative dose of corticosteroids in the immediate post-KT period ($r = -0.32$; $p = 0.007$).

Table I. Evolution of analytical parameters related to bone-mineral metabolism

	Missing (*n*)	Baseline	6 months	12 months	*p*-value (Baseline vs 6m)	*p*-value (6 m vs 12 m)	*p*-value (Baseline vs 12 m)
Phosphate (mmol/L)	4/2/13	1.75 ± 0.5	1.08 ± 0.20	1.13 ± 0.22	< 0.0001	< 0.01	< 0.0001
Calcium (mmol/L)	8/3/13	2.25 ± 0.18	2.41 ± 0.11	2.40 ± 0.11	< 0.0001	ns	< 0.0001
Magnesium (mmol/L)	31/31/37	1.03 ± 0.16	0.68 ± 0.11	0.72 ± 0.11	< 0.0001	ns	< 0.0001
PTH (ng/L)	7/24/29	239 ± 124	91 ± 40	92 ± 40	< 0.0001	ns	< 0.0001
25(OH)D_3 (ng/mL)	13/16/24	19.3 ± 10	30.8 ± 11	29.3 ± 9	< 0.0001	ns	< 0.0001
Creatinine (µmol/L)	9/6/18	516 ± 163	145 ± 52	141 ± 47	< 0.0001	ns	< 0.0001
eGFR CKD-EPI (mL/min/1.73 m^2)	3/3/12	9.5 ± 3.5	46.3 ± 18.8	45.7 ± 19.4	< 0.0001	ns	< 0.0001

Data are expressed as mean ± standard deviation or median (IQR). PTH: parathyroid hormone; eGFR: estimated glomerular filtration rate; 25(OH)D_3: 25-hydroxyvitamin D. ns: not significant. Missing: number of patients with unavailable data at baseline/6 months/12 months post-transplant.

Table II. Evolution of bone turnover biomarkers

	n	Immediate post-KT	6 months	12 months	*p*-value
BALP (µg/L)	67/55/65	9.03 ± 3.95	11.96 ± 4.46	11.18 ± 4.71	< 0.001
P1NP (µg/L)	65/56/66	48.4 ± 35.7	86.2 ± 50.4	64.6 ± 42.6	< 0.001
TRAP5b (U/L)	70/59/68	4.25 ± 2.52	3.40 ± 2.23	3.61 ± 2.01	0.085

BALP: bone alkaline phosphatase (normal values for men: < 20 µg/L, premenopausal women < 14 µg/L and postmenopausal women < 22 µg/L) (25); P1NP: procollagen type 1 N-terminal propeptide (normal values for men 23-95 µg/L, premenopausal women 15-59 µg/L and postmenopausal women 20-76 µg/L); TRAP 5b: tartrate-resistant acid phosphatase 5b (normal value < 6.5 U/L) (26).

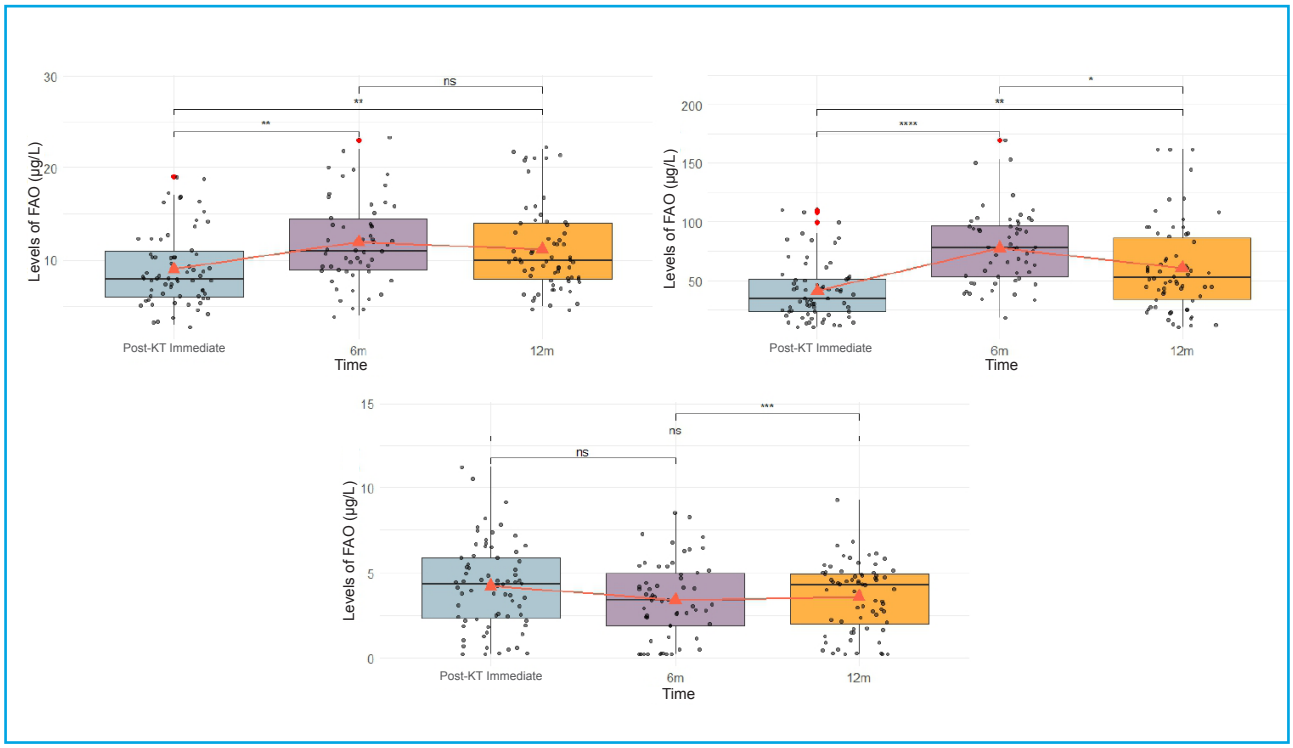

Figure 1. Evolution of MDROs during the first-year post-transplant (n.s.: not significant; ****$p < 0.00001$; ***$p < 0.0001$; **$p < 0.001$; *$p < 0.01$).

A significant increase in BALP and P1NP was observed at 12 months post-KT compared to the immediate post-KT period, with the increase being more pronounced during the first 6 months. No significant changes were observed in TRAP 5b at 12 months. BALP and P1NP levels, compared to the immediate post-KT period, increased by 23 % and 118 % at 6 months, and by 28 % and 62 % at 12 months, respectively. TRAP5b only increased by 3.3 % at 6 months and 33 % at 12 months.

Differences between patients treated *versus* not treated with anti-resorptive agents

A total of 32 patients (26.2 %) received anti-resorptive treatment (6 denosumab, 26 bisphosphonates) at 65 ± 42 days post-KT. The main clinical and analytical characteristics are shown in table III. The mean age of treated patients was 60 years, 47 % were women, and 15.6 % were diabetic. The behavior of bone formation and resorption biomarkers was different in patients treated with anti-resorptive agents compared to untreated patients (Fig. 2). Unlike the untreated group, treated patients did not show an increase in BALP and P1NP at 12 months. BALP and P1NP levels were significantly lower in the treated group at 12 months, while no differences were observed between groups in the immediate post-KT period (pre-treatment). TRAP 5b levels at 12 months were also significantly lower in the treated group. However, no significant differences were observed in their evolution when comparing both groups. In the treated group compared to the untreated group, the percentage of women was higher, and they had a lower BMI. No significant differences were observed in the estimated glomerular filtration rate (eGFR), as well as in age, dialysis time, or treatment with calcium or vitamin D.

Multivariate analysis

Multivariate models show that the changes observed at 12 months in BALP and P1NP with respect to the immediate post-KT period remain independent of age, sex, diabetes, corticosteroid dose, eGFR, and baseline PTH. However, a higher immediate post-KT value of BALP (beta: -0.80; $p < 0.001$) and P1NP (beta: -0.99; $p = 0.001$) is related to a smaller change at 12 months in both markers. Similarly, patients who received treatment show a smaller change in both cases (β: -2.83; $p = 0.049$) and (β: -20.33; $p = 0.049$) respectively.

Table III. Characteristics of treated and untreated patients

	All (*n* = 122)	No anti-OP treatment (*n* = 90)	Anti-OP treatment (*n* = 32)	*p*-value
Age (years)	58 ± 13	57 ± 12.3	60 ± 13	0.886
Female sex (*n*, %)	37 (30.1 %)	22 (24.4 %)	15 (46.9 %)	0.032
BMI (kg/m^2)	25.6 ± 3.8	26.1 ± 4	24.2 ± 2.6	0.006
DM (*n*, %)	28 (22.8 %)	22 (24.4 %)	5 (15.6 %)	0.433
CKD HD (*n*, %)	65 (52.8 %)	47 (52.2 %)	17 (53.1 %)	0.574
Time on dialysis (months)	25.3 ± 15.9	23.6 ± 14.4	24 ± 14.8	0.836
History of fragility fracture (*n*, %)	13 (10.7 %)	5 (38.5)	8 (61.5)	0.04
Lumbar spine T-score (SD)	-0.7 ± 1.9	-0.2 ± 1.8	-1.6 ± 1.5	< 0.001
Lumbar osteoporosis (*n*, %)	17 (15.2 %)	7 (8.6 %)	10 (32.3 %)	0.006
Femoral neck T-score (SD)	-1.5 ± 1.2	-1.1 ± 1.0	-2.4 ± 1.2	< 0.001
Femoral neck osteoporosis (*n*, %)	25 (22.7 %)	13 (16 %)	12 (41.4 %)	< 0.001
Total hip T-score (SD)	-1.3 ± 1.3	-0.8 ± 1.1	-2.2 ± 1.0	< 0.001
Total hip osteoporosis (*n*, %)	17 (15.3 %)	9 (11.1 %)	8 (26.7 %)	< 0.001
Baseline eGFR CKD-EPI (mL/min/1.73 m^2)	9.5 ± 3.5	9.6 ± 3.5	9.03 (3.55)	0.474
12-month eGFR CKD-EPI (mL/min/1.73 m^2)	45.7 ± 19.4	45.2 ± 19.4	46.9 ± 19.4	0.904
Baseline calcium (mmol/L)	2.25 ± 0.18	2.23 ± 0.17	2.31 ± 0.19	0.017
12-month calcium (mmol/L)	2.40 ± 0.11	2.40 ± 0.11	2.41 ± 0.09	0.271
Baseline 25(OH)D_3 (ng/mL)	19.65	19.40	20.55	0.473
12-month 25(OH)D_3 (ng/mL)	29.3 ± 9	28.6 ± 9.7	31.1 ± 7	0.230
Baseline PTH (ng/L)	239 ± 124	241 ± 126	239 ± 119	0.997
12-month PTH (ng/L)	92 ± 40	94 ± 43	85 ± 31	0.541
12-month treatment (*n*, %)				
Native vitamin D*	85 (69.1 %)	63 (70.0 %)	22 (68.8 %)	0.447
Calcium	75 (61.0 %)	55 (61.1 %)	20 (62.5 %)	0.744
Cinacalcet	12 (9.8 %)	5 (5.6 %)	12 (37.5 %)	0.04
Cumulative corticosteroid dose (g)	2.7 ± 0.3	2.7 ± 0.3	2.6 ± 0.3	0.642

*Data are expressed as mean ± standard deviation or median (IQR). 1 patient was excluded due to missing data on anti-resorptive treatment. BMI: body mass index; DM: diabetes mellitus; CKD: chronic kidney disease; HD: hemodialysis; eGFR: estimated glomerular filtration rate; 25(OH)D_3: 25-hydroxyvitamin D; PTH: parathyroid hormone; OP: osteoporosis. *Calcidiol or ergocalciferol.*

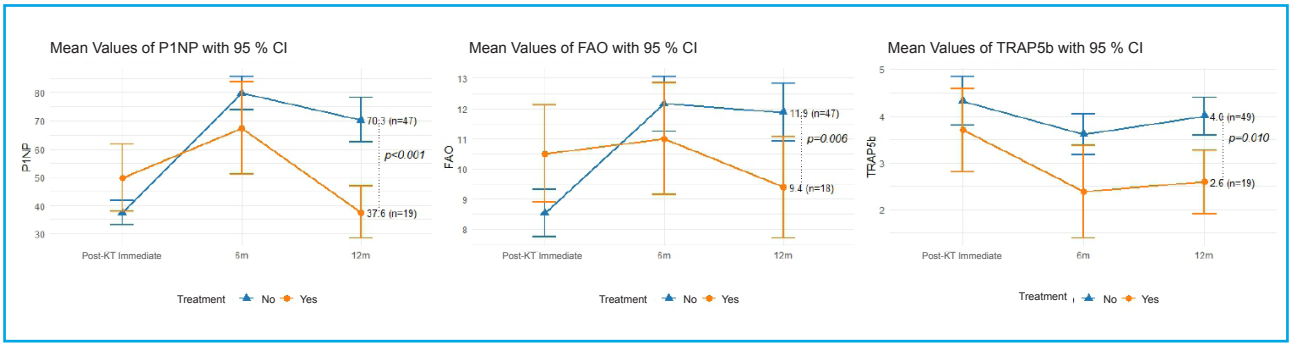

Figure 2. Comparison of MDRO progression between patients treated with antiresorptives and those untreated. In the untreated group, a significant increase was observed at 12-months in ALP ($p < 0.001$) and P1NP levels ($p < 0.0001$), along with a significant decrease in TRAP5b ($p < 0.001$). In the treated group, the changes were not statistically significant.

DISCUSSION

The main result of the study is that the formation biomarkers, BALP and P1NP, increase during the first year post-KT, especially during the first 6 months, with no significant changes in TRAP5b. PTH, however, shows a significant decrease during the first semester and then remains stable during the second. Anti-resorptive treatment decreased BTMs at 12 months post-KT.

The abrupt decrease in PTH levels we observed during the first 6 months post-KT is due to the resolution of hyperparathyroidism due to improved calcitriol production upon restoration of renal mass (increasing 1-alpha hydroxylase activity and decreasing FGF-23). Our results are consistent with findings in previous cohorts that show a similar evolution of PTH during the first year post-KT (11,12). Evenepoel et al. observed a 59.5 % decrease in PTH values, very similar to the reduction we observed (61.6 %) (11). The persistence of post-KT hyperparathyroidism has been associated with a deterioration of cortical bone, which could explain the high rates of peripheral skeletal fractures in this population (13). Similarly, greater decreases in PTH levels have been associated with an improvement in BMD during the first year post-KT (14).

BALP is released by osteoblasts during the mineralization process, and P1NP is a fragment released when collagen is deposited in the bone matrix, so both are considered bone formation markers. TRAP5b is an enzyme originating from osteoclasts and is very specific for bone resorption. In our study, we observed an overall increase in bone formation markers at one-year post-KT, with this increase being particularly notable during the first 6 months. However, we did not observe large differences in TRAP5b. The observed behavior of BTMs does not coincide with other previously published series where a decrease in bone turnover biomarkers is observed during the first year post-KT (11,15). The difference we observed compared to other studies may be due to the effect of corticosteroids. The initial determination of BTMs in our study was performed 48 hours post-KT, where patients had already received an estimated mean dose of 300 mg of corticosteroids, while in the Belgian study, it was performed pre-KT or on the same day of the intervention, when they had not yet received corticosteroids. The main mechanism by which corticosteroid therapy is associated with a high risk of fracture is the suppression of bone formation. The decrease in bone formation begins on the first day of drug administration and is dose-dependent, being maximal during the first week; however, no changes in bone resorption are observed, leading to an imbalance in remodeling (16,17). The effect of corticosteroids on the expression of formation BTMs may have influenced the levels of BALP and P1NP, being lower in our study compared to other series (BALP: 9 vs 20.9 µg/L, P1NP: 48.4 vs 79 µg/L) (15). In contrast, the values at 12 months, when the cumulative dose of corticosteroids is similar in both series, no longer differed as much, being: 11.2 vs 17.4 µg/L for BALP and 64.6 vs 64.3 µg/L for P1NP (15). On the other hand, in the study by Bonani et al., where P1NP and BALP were analyzed 15 days after KT (when 100 % of patients were already on corticosteroid treatment), the observed values were more similar to those found in our study (18). Similarly, Tada et al. in the TOMORROW study demonstrated that reducing the dose of glucocorticoids in patients with rheumatoid arthritis improved osteocalcin levels (a bone formation marker) but not NTX-1 (N-terminal telopeptide of collagen I) (a bone resorption marker) (19). We can conclude that corticosteroids possibly decreased BTMs early and significantly, especially those of bone formation.

The evolution of BTMs in the group of patients on anti-resorptive agents was different from that in the untreated group. In treated patients, bone formation and resorption markers did not show an increase during the follow-up period and were significantly lower compared to untreated patients at 12 months. The two groups were balanced for age, PTH level, and renal function. A previous study that evaluated the efficacy of low doses of pamidronate in the immediate post-KT period observed that P1NP levels normalized by the third month of treatment and remained stable at one year, compared to a placebo group that recovered levels by increasing markedly during the second semester (20). Bonani et al. also analyzed the effect of anti-resorptive treatment at one year, but only with denosumab (n = 46) in *de novo* KT recipients, in a randomized and placebo-controlled study (n = 44). In the treatment group, formation (P1NP, BALP) and resorption (CTX) biomarkers also decreased compared to the control group (18). Anti-resorptive treatments inhibit osteoclast activity and reduce bone resorption, restoring the balance in bone remodeling. The cross-talk between osteoblasts and osteoclasts (21) would explain why bone formation, reflected in a decrease in BALP and P1NP biomarkers, also decreases during anti-resorptive treatment. Osteoclasts recruit osteoprogenitors through factors such as SIP (sphingosine kinase) or BMP6 (bone morphogenetic protein-6), which will stimulate bone formation (21). The decrease in bone resorption will cause a rapid reduction in bone formation due to a lower release of these factors (SIP, BMP6). The increase in BMD during anti-resorptive treatment could be due to a more pronounced decrease in resorption than the decrease in bone formation, and this suppression of bone turnover would also increase the time for mineralization, favoring the increase in density (22). The decrease in BTM expression has been related to an increase in BMD. In postmenopausal women treated with alendronate, the greater the decrease in short-term BTMs, the greater the long-term increase in BMD (23). In the nephrological setting, Jørgensen et al. analyzed a cohort of 209 *de novo* KT recipients without anti-resorptive treatment and observed that patients who lost more BMD at 12 months post-KT had

higher levels of BALP, P1NP, and TRAP5b compared to those who had maintained stable BMD (15). The determination of biomarkers at the start of anti-resorptive treatment could predict the therapeutic response.

One of the strengths of the study is the determination of BTMs without renal clearance, such as BALP or TRAP5b; however, P1NP was determined in its monomeric form. P1NP is a collagen fragment released when it is deposited in the bone matrix. Monomeric fragments of P1NP accumulate in CKD, so it is recommended in these cases to measure the trimeric or intact form, which is not modified by renal clearance. The patients in our study, however, showed a recovery of renal function, with an eGFR of 46 ± 19 mL/min/1.73 m^2 at 6 months post-KT, so the accumulation should be lower, and in any case, the eGFR was not different between the treated and untreated patient groups.

There is not much experience in the use of biomarkers in kidney transplant recipients, and even less on their behavior after the start of anti-resorptive treatment (18,20). In the nephrological field, they are emerging as a potential alternative to bone biopsy for the evaluation of renal osteodystrophy, being useful in clinical practice to rule out the presence of high or low bone turnover (9). Recently, a European consensus document for the diagnosis and management of osteoporosis in CKD 4-5D has been published, in which the authors suggest using bone biomarkers for the diagnosis and monitoring of treatment in patients with CKD, particularly those without renal clearance, such as BALP, P1NP in its trimeric form, and TRAP5b (24). Our study is the first to evaluate the evolution of biomarkers in a subgroup of patients treated mainly with oral bisphosphonates, although the main limitation is the small sample size. The baseline sample of BTMs was collected a few days after KT, unlike other series in which it was obtained previously. Due to the effect of corticosteroids, the baseline time point in our study would not be directly comparable with that of other studies. However, this allows us to observe the effect of corticosteroids on the expression of BTMs. Another limitation is that the value of change in BTMs has been analyzed without considering the minimum significant change, so it cannot be ruled out that the variability in the expression of BTMs has influenced the results. Most patients included in the study were male (70 %), however, despite the high rate of hypogonadism in CKD and patients treated with corticosteroids, we do not have that data, which represents another limitation for the study. The percentage of patients eligible for anti-osteoporotic treatment in our population would be estimated to be much higher. However, in the nephrological setting and specifically in kidney transplantation, the initiation of anti-fracture treatment may be limited by a number of factors (especially during the first months post-KT) such as delayed graft function, the concurrence of other acute pathologies, persistent hyperparathyroidism, the risk of hypocalcemia associated with the initiation of denosumab, or lack of therapeutic adherence associated with polypharmacy.

In conclusion, during the first year of KT, formation biomarkers increase, especially during the first semester, considering that we start from a reduced baseline level due to the effect of the initial high dose of corticosteroids. PTH shows a significant decrease during the first semester and then remains stable during the second semester. Anti-resorptive treatment decreases BTMs (formation and resorption) 1-year post-KT, without observing changes in eGFR or PTH compared to untreated patients. BTMs provide information on the effect of anti-resorptive treatment during the first year of KT, as well as on the possible influence of short-term corticosteroid treatment.

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Original

Impact of PTHrP on RAW 264.7 macrophage proliferation and polarization

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Abstract

Background: bone, a mineralized connective tissue, is in constant remodeling thanks to the action of osteoclasts (bone resorption) and osteoblasts (bone formation) in a process regulated by osteocytes, which act as mechanosensors. In addition, the immune system plays an essential role in bone regeneration, highlighting the importance of the interaction between bone cells and immune cells. In particular, macrophages can be polarized towards a proinflammatory M1 or anti-inflammatory or regenerative M2 phenotype, the latter being relevant in tissue repair.

Material and methods: in this context, it has been observed that PTHrP acts as a cytokine that regulates cell proliferation and differentiation, especially in cells involved in bone regulation. In this study, we have investigated how PTHrP (1-37) affects the proliferation and polarization of RAW 264.7 macrophages towards an M1 or M2 phenotype, as well as its impact on PTH1R receptor expression and osteoclastic markers.

Results and conclusion: the results show that PTHrP does not modify macrophage proliferation or polarization, but reduces the expression of PTH1R in the M2 phenotype and that of certain osteoclastic markers. This suggests a modulatory role of PTHrP in the osteoclastic capacity of precursors, indicating a possible impact on bone remodeling and immune regulation.

Keywords:
PTHrP.
Macrophages.
Polarization.
Proliferation.
Osteoclastogenesis.
PTH1R.

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INTRODUCTION

Bone is a mineralized connective tissue that presents four main cell types: osteoblasts, bone lining cells, osteocytes, and osteoclasts (1). It plays very important roles in the organism, such as locomotion, support and protection of soft tissues, hematopoiesis, and calcium and phosphate storage (2). Despite its inert appearance, bone is a dynamic and metabolically active tissue that is constantly undergoing remodeling, suffering bone resorption by osteoclasts and bone formation by osteoblasts (3). Furthermore, there is evidence that osteocytes act as mechanosensors that regulate this process (4).

Bone and immune cells coexist in the bone marrow cavity and share various regulatory molecules. In addition, the immune system plays an important role in tissue repair and regeneration, thus determining the bone tissue's capacity to regenerate. Hence the importance of osteocyte communication with its entire bone microenvironment, including monocytes, osteoclasts, osteoblastic precursors, and T lymphocytes (5).

Macrophages are immune cells that participate in various physiological and pathological processes such as organ development, acute and chronic inflammation, and tissue homeostasis and remodeling (6). Macrophages can be phenotypically polarized according to the stimulus received into two large groups: classically activated macrophages (M1), with pro-inflammatory effects; and alternatively activated macrophages (M2), with effects on immune regulation and tissue remodeling (7-9).

Type I macrophages (M1) are mainly induced by Toll-like receptor (TLR) ligands such as bacterial lipopolysaccharide (LPS), producing inflammatory cytokines and presenting high levels of TNF-α and iNOS (10); while polarization to M2 macrophages is induced by cytokines such as IL-4 and IL-13, producing cytokines that promote tissue anabolism and presenting high levels of CD206 (9,11).

Osteoclasts are multinucleated cells that derive from mononuclear cells of the hematopoietic lineage (monocytes/macrophages), whose differentiation is induced by various factors, including macrophage colony-stimulating factor (M-CSF), produced by osteoprogenitor and osteoblast mesenchymal cells (12), and RANKL, secreted by osteoblasts, osteocytes, and stromal cells (13). The RANK/RANKL interaction stimulates the expression of key factors in osteoclastogenesis, such as nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), and dendritic cell-specific transmembrane protein (DC-STAMP). NFATc1, in collaboration with the transcription factors PU.1, c-FOS, and MITF, regulates the expression of osteoclast-specific genes, such as cathepsin K and tartrate-resistant acid phosphatase (TRAP), essential for osteoclastic activity (14). It is important to note that macrophages and osteoclasts share a common origin, both originating from the same hematopoietic precursor (15).

Parathyroid hormone-related protein (PTHrP) is a cytokine with paracrine and/or autocrine functions, among which are the control of tissue and organ development, proliferation, differentiation, and cell survival through its interaction with the PTH/PTHrP type I receptor (PTH1R) (16,17). It has been shown that cells involved in bone regulation, such as osteoprogenitors, osteoblasts, osteocytes, T cells, and macrophages, express PTH1R, making them sensitive to stimulation with PTHrP (18-20). Although the role of the PTHrP/PTH1R system in cells of the monocyte/macrophage lineage is not clear, the presence of the receptor in these cells (21,22) suggests a role in the regulation of the immune system, potentially modulating inflammatory responses, inflammation, and migration (20).

Numerous studies have demonstrated the importance of macrophages in tissue repair and, especially in bone, M1 and M2 polarization is crucial for regeneration (6,23). In addition, monocytes are crucial osteoclastic precursors for bone resorption (24). Based on this background, we aimed to analyze whether PTHrP (1-37) affects macrophage proliferation, M1 and M2 polarization of macrophages, as well as the expression of the PTH1R receptor and osteoclastic markers.

MATERIALS AND METHODS

CELL CULTURES

Murine RAW 264.7 macrophages were cultured in DMEM medium (41966-029, Gibco) supplemented with 10 % fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 µg/mL) in a humidified incubator with 5 % CO_2 at 37 °C. For the viability and proliferation assay, RAW 264.7 cells were seeded at a density of 25,000 cells/cm^2 in conventional culture plates. Once the cells were seeded, stimulation with 100 nM of the PTHrP (1-37) peptide and with 100 ng/mL of LPS or 20 ng/mL of IL-4 was performed simultaneously. After treatment, the cells were maintained in culture for 24, 48, and 72 hours. For the gene expression assay, the cells were seeded at a density of 30,000 cells/cm^2 in conventional culture plates. The cells were maintained in culture until they were almost completely confluent, at which point stimulation was performed for 24 hours with 100 ng/mL of LPS or 20 ng/mL of IL-4 to induce macrophage polarization to an M1 or M2 phenotype, respectively. Subsequently, before the M1 or M2 polarization was complete, treatment with PTHrP (1-37) (Bachem, Bubendorf, Switzerland) at a concentration of 100 nM was carried out for 6 hours, with both treatments ending simultaneously. After this time, RNA extraction was performed with trizol (Ambion, Life Technologies).

CELL VIABILITY AND PROLIFERATION ASSAYS

After the time periods described in the "Cell cultures" section, the cells were scraped with a scrapper, and both adherent and non-adherent cells were collected in a tube and resuspended in culture medium for subsequent staining with trypan blue. The number of live cells (unable to take up trypan blue because they have an intact membrane) was then counted using a Neubauer chamber, obtaining a cell proliferation curve.

QUANTITATIVE REAL-TIME PCR (RT-qPCR)

Total RNA extraction was performed using the guanidinium thiocyanate-phenol-chloroform separation method. The amount of RNA obtained was then quantified with the NanoDrop 2000 (ThermoFisher Scientific), and reverse transcription (from 2 µg of RNA) was carried out to obtain complementary DNA (cDNA) using the high capacity cDNA reverse transcription kit (Applied Biosystem, Grand Island, NY) in an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany) following the manufacturer's instructions. Subsequently, qPCR was performed on the QuantStudio™ 5,384-Well Block (ThermoFisher Scientific) using SYBR Premix ex Taq (Takara, Otsu, Japan) and specific primers for *Tnf* (TNF-α), *Nos*2 (iNOS), *Mrc*1 (CD206), *Pth1r* (PTH1R), *Acp*5 (TRAP), *Nfatc*1 (NFATc1), and Tnfrsf11a (RANK), using *18S* (18S) as a housekeeping gene. The protocol used consisted of an initial reaction of 10 minutes at 95 °C, followed by 45 cycles of 15 seconds at 95 °C, 15 seconds at 60 °C, and 15 seconds at 72 °C; then, to obtain the primer dissociation curve, the temperature was increased to 95 °C, lowered to 65 °C for 15 seconds, and raised again to 95 °C. Finally, the temperature was decreased to 40 °C for 30 seconds. Changes in gene expression have been represented as target gene expression levels relative to their control. For this, the comparative relative quantification method using ΔΔCt (25) was employed. This methodology considers 100 % amplification efficiency during qPCR and the same efficiency for both genes. The sequences of the primers used are shown in table I.

Table I. Primers used in RT-qPCR

Gene	Forward 5'-3'	Reverse 5'-3'
18S	ATGCTCTTAGCTGAGGTGCCCG	ATTCCTAGCTGCGGTATCCAGG
Tnf	AGGCACTCCCCCAAAAGATG	TGAGGGTCTGGGCCATAGAA
Nos2	CCTGCTTTGTGCGAAGTGTC	CCCTTTGTGCTGGGAGTCAT
Mrc1	CCACAGCATTGAGGAGTTTG	ACAGCTCATCATTTGGCTCA
Pth1r	TGAAGGACGCTGTGCTCTACTC	AGTAGAGGAAGAAGGTCACGGC
Acp5	CACGAGAGTCCTGCTTGTC	AGTTGGTGTGGGCATACTTC
Nfatc1	TCATCCTGTCCAACACCAAA	TCACCCTGGTGTTCTTCCTC
Rank	GGACAACGGAATCAGATGTGGTC	CCACAGAGATGAAGAGGAGCAG

STATISTICAL ANALYSIS

Data are expressed as means ± standard deviations (SD), and statistical analyses were performed with GraphPad Prism version 9.1.0. For gene expression analysis, significant differences were sought in each treatment. These differences were analyzed using non-parametric analysis of variance (Kruskal-Wallis) followed by the Mann-Whitney test. A $p < 0.05$ was considered significant. In the case of the heatmap, the Benjamini, Krieger, and Yekutieli two-stage linear step-up procedure was applied to control the FDR (false discovery rate) with a q-value < 0.1.

RESULTS

CELL PROLIFERATION

We wanted to check how treatment to induce polarization towards an M1 or M2 phenotype, with LPS and IL-4 respectively, affects the proliferation of murine RAW 264.7 macrophages. Using the vital dye trypan blue, it was observed that LPS induced a significant increase in proliferation compared to the control; however, IL-4 did not modify the proliferation of RAW 264.7 cells (Fig. 1A). We also studied whether treatment with PTHrP (1-37) affected cell proliferation in synergy with LPS and IL-4. In the absence of polarization factors, PTHrP (1-37) did not induce significant changes in the number of live cells that proliferate in the studied time period (Fig. 1B). In macrophages stimulated with LPS or IL-4, PTHrP (1-37) did not induce changes in the number of live cells compared to their stimulation with LPS and IL-4, respectively (Fig. 1 C and D).

EFFECT OF PTHrP (1-37) ON M1 AND M2 POLARIZATION

It was verified that LPS and IL-4 induced polarization to an M1 and M2 phenotype in RAW 264.7 cells, respectively. Treatment with LPS stimulated the production of TNF-α and iNOS (M1 markers), while it decreased the expression of CD206 (M2 marker). Conversely, stimulation with IL-4 increased the expression of CD206; while the expression of TNF-α decreased. Treatment with PTHrP (1-37) did not significantly affect M1 or M2 polarization; however, it significantly reduced the expression of CD206 in cells not treated with LPS or IL-4 (Fig. 2 A-C).

Afterwards, we evaluated how macrophage polarization and treatment with PTHrP (1-37) affected the expression of the PTH1R receptor. It was observed that M1 and M2 polarization produced a decrease

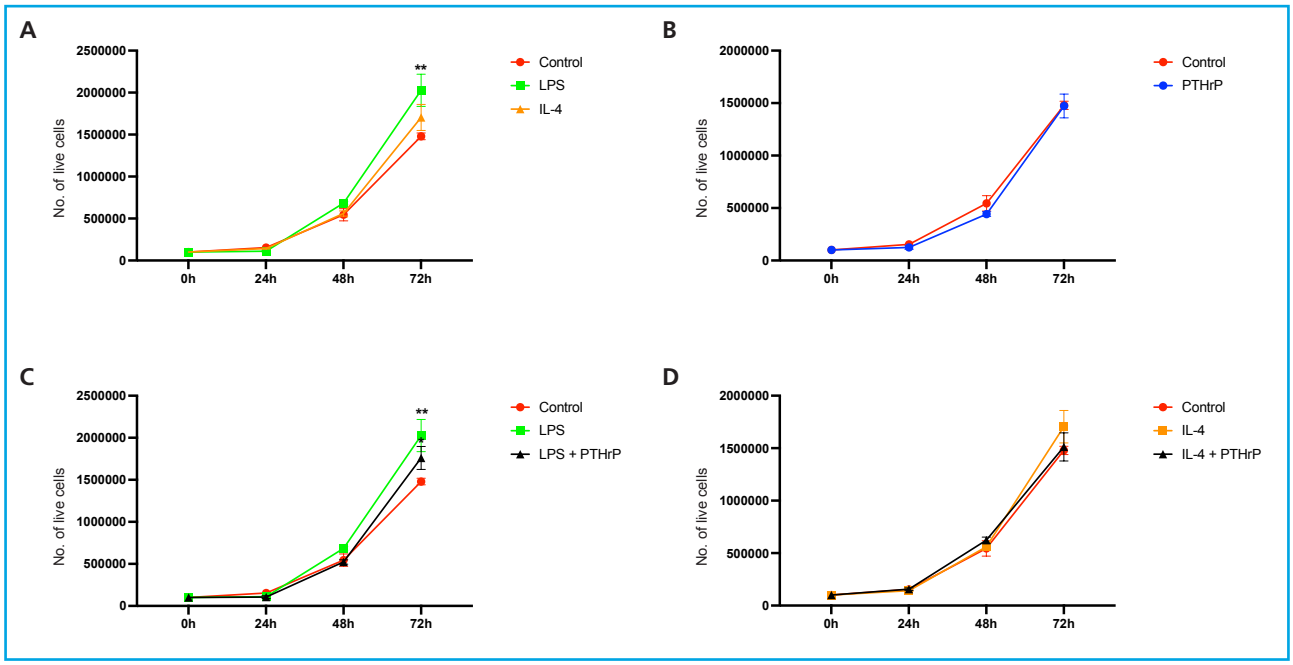

Figure 1. PTHrP (1-37) does not induce significant changes in the cell proliferation of M1 and M2 macrophages. Number of live cells under control conditions, LPS treatment, and IL-4 treatment A. Number of live cells under control conditions and PTHrP (1-37) treatment B. Number of live cells under control conditions, LPS treatment, and LPS + PTHrP (1-37) treatment C. Number of live cells under control conditions, IL-4 treatment, and IL-4 + PTHrP (1-3 treatment D. The results are the means ± SD of triplicates from three different experiments. *$p < 0.05$ vs control; **$p < 0.01$ vs control.

in PTH1R expression. However, the decrease in the M1 phenotype was more pronounced (up to 4 times). In addition, PTHrP (1-37) induced a decrease in receptor expression, both under basal conditions and in the M2 phenotype, but did not affect expression in the M1 phenotype (Fig. 2D).

Subsequently, the effects of polarization and treatment with PTHrP (1-37) on the expression of the osteoclastic markers TRAP, NFATc1, and RANK were analyzed. Polarization of cells towards an M1 phenotype decreased the expression of osteoclastic markers. In contrast, polarization towards an M2 phenotype increased the expression of TRAP without significantly affecting the expression of NFATc1 and slightly reducing the expression of RANK. In addition, treatment with PTHrP (1-37) decreased the expression of the osteoclastic markers NFATc1 and RANK in M2 macrophages without affecting M1 macrophages (Fig. 2 E-G).

We also generated a heatmap in which the correlation in the expression of the studied genes is represented by colors (positive correlation 0 to 1 [blue] or negative correlation 0 to -1 [red]) (Fig. 3). A positive correlation is observed between osteoclastic markers and the M2 phenotype of macrophages, while there is a negative correlation with the M1 phenotype. Furthermore, there is also a negative correlation in the expression of PTH1R with the M1 phenotype, but positive with the M2 phenotype. Finally, in the case of osteoclastic markers, these correlate positively with the expression of the receptor, so that when PTH1R expression increases, there is an increase in the expression of osteoclastic markers.

DISCUSSION

Bone is a dynamic organ that undergoes a remodeling process directed by osteoclasts, osteoblasts, and osteocytes. During bone remodeling, damaged bone is removed by osteoclasts and replaced by osteoblasts. This remodeling process involves the formation of osteoclasts from their precursors of the monocyte-macrophage lineage (3).

The PTH1R receptor is expressed in osteoblasts, osteocytes (26,27), and other cells present in the bone marrow such as monocytes and T cells (21,28). In the present work, we demonstrate that the RAW 264.7 macrophage cell line expresses the PTH1R receptor, and that after stimulation by its ligand PTHrP (1-37), the expression of genes related to polarization and osteoclastic differentiation is modulated without affecting cell proliferation.

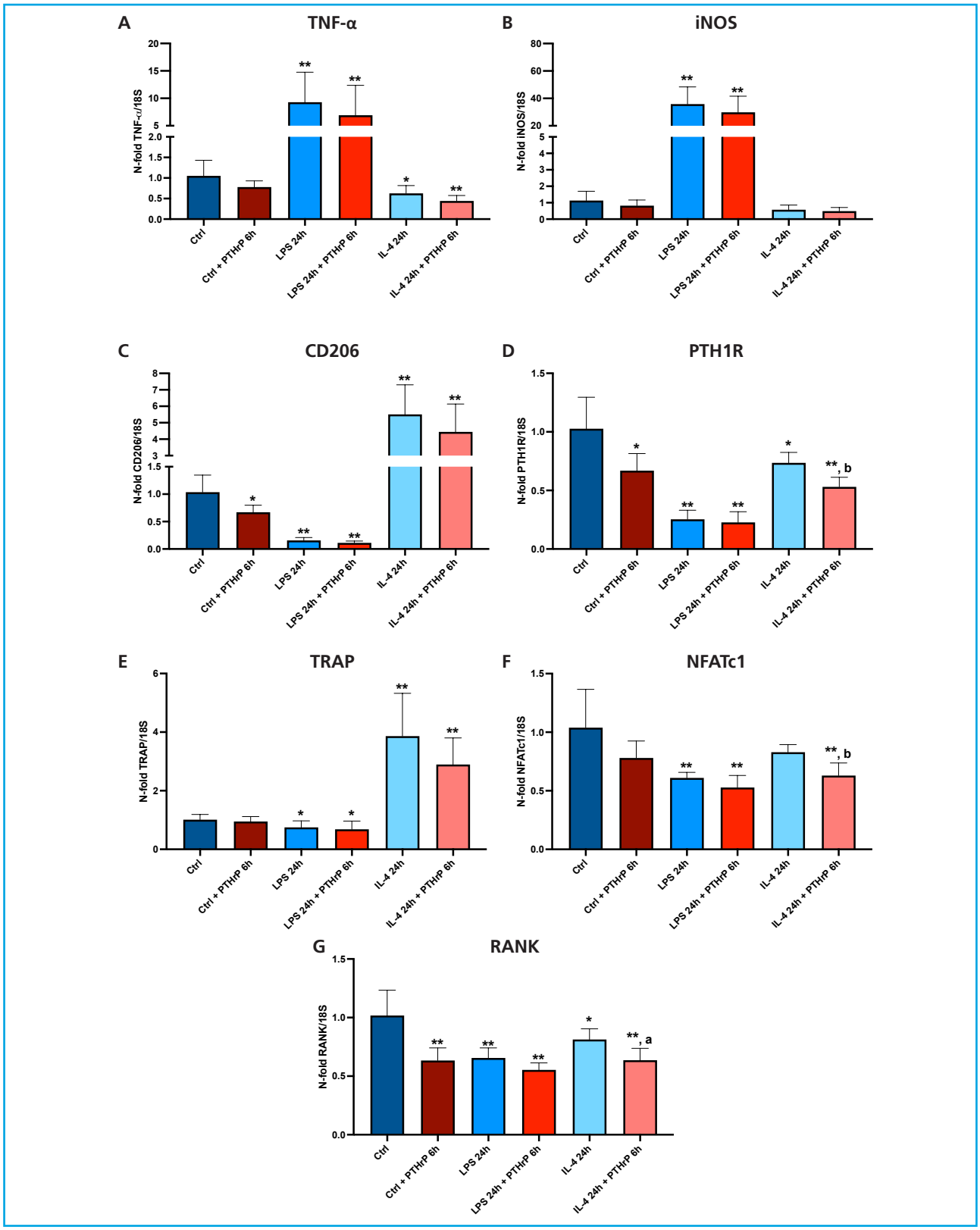

Figure 2. Relative expression of TNF-α (A), iNOS (B), CD206 (C), PTH1R (D), TRAP (E), NFATc1 (F), and RANK (G). Cells were treated with LPS and IL-4 (100 ng/mL and 20 ng/mL, respectively) for 24 hours, and for the last 6 hours, they were stimulated with PTHrP (1-37) 100 nM. The results are the means ± SD of triplicates from two different experiments. *$p < 0.05$ vs control; **$p < 0.01$ vs control; ***$p < 0.001$ vs control; $^{a}p < 0.05$ vs stimulus; $^{b}p < 0.01$ vs stimulus.

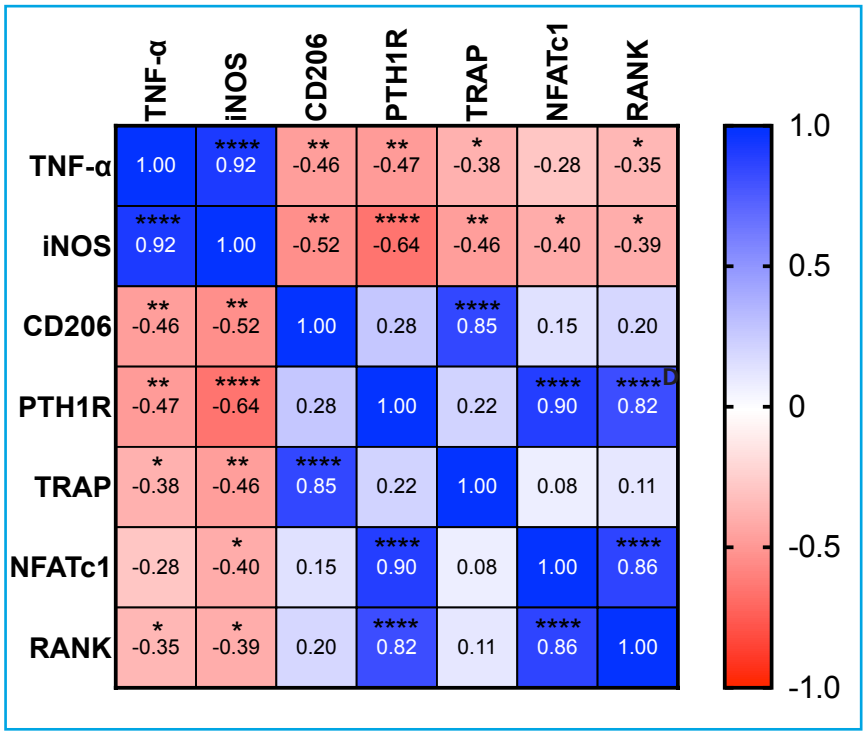

Figure 3. Heatmap of the positive or negative correlation between the expression of TNF-α, iNOS, CD206, PTH1R, TRAP, NFATc1, and RANK. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. The Benjamini, Krieger, and Yekutieli two-stage linear step-up procedure was applied to control the FDR (false discovery rate) with a q-value < 0.1.

Our results indicate that stimulation with LPS induces a significant increase in the proliferation of RAW 264.7 cells. In the study carried out by Jiao et al. in 2016, it is demonstrated that LPS stimulates the proliferation of monocytes/macrophages through the regulation of the TDAG51 protein, which plays an essential role in cell cycle progression (29); and, furthermore, it has been shown that IL-4 increases the survival of differentiated mouse basophils in vitro through signaling independent of phosphoinositide 3-kinase (PI3K) transcription (30), so it could have the same function in the monocyte/macrophage lineage.

Results also indicate that the polarization of these cells towards a regenerative M2 phenotype in turn induces the expression of osteoclastic markers. It is observed that in macrophages polarized to an M2 phenotype, there is a positive correlation in the gene expression of the M2 marker CD206 with the osteoclastic markers TRAP, NFATc1, and RANK. However, in M1 macrophages, the correlation with osteoclastic markers is negative. The study presented by Yu et al. in 2009 shows that IL-4 is capable of increasing the gene expression of TRAP in RAW 264.7 cells on its own (31). In addition, $F4/80^{+}CD206^{+}$ M2 synovial macrophages present in rheumatoid arthritis may be new osteoclastic precursors and contribute significantly to bone changes, as they have been shown to highly express RANK and can be activated by RANKL and M-CSF to acquire osteoclast markers and bone resorption function (32). Osteoclasts are multinucleated cells of the monocyte/macrophage lineage that require M-CSF and RANKL for their differentiation. Some studies suggest that M2 macrophages can influence the expression of osteoclastic markers such as TRAP. For example, in a study on the regeneration of dental alveoli after extraction, it was observed that M2 macrophages promoted the differentiation of osteoprogenitor cells through the secretion of TGF-β and, in the later stages, TRAP-positive osteoclasts were detected (33), suggesting a connection between M2 macrophages and osteoclast formation. Furthermore, there is other evidence suggesting the possible fusion of M2 macrophages with osteoclasts, which could promote the secretion of osteogenic cytokines, thus stimulating osteogenic differentiation and bone formation in osteoinductive materials (34). Therefore, there could be a relationship between the increased expression of TRAP and the M2 polarization of macrophages. However, it is important to note that other studies indicate that the secretion of TNF-α and IL-1β by M1 macrophages is capable of maturing osteoclastic precursors for osteoclast formation (35); while M2 macrophages could inhibit osteoclast development by secreting IL-4 and IL-10 (36). This could suggest that, although M2 macrophages derived from RAW 264.7 cells express high levels of TRAP, they do not fully differentiate into an osteoclast.

Finally, PTHrP (1-37) induces a significant decrease in the expression of PTH1R in the RAW 264.7 macrophage cell line, which could show a negative regulation induced by the excess of receptor agonist.

Our findings suggest that stimulation with LPS and IL-4 can affect the proliferation of M1 and M2 macrophages, as well as the expression of osteoclastic markers, thus modifying the osteoclastogenic capacity of these precursors. However, PTHrP (1-37) does not modulate the proliferation of RAW 264.7 cells, although it does have effects on the expression of characteristic osteoclast markers. Therefore, more studies are needed to address the role of PTH1R in these cells to clarify the possible effects of its ligands PTH and PTHrP in the monocyte-macrophage lineage.

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Original

Evaluation of calcium intake in postmenopausal women treated with supplements in primary care

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Abstract

Background: calcium and vitamin D requirements in postmenopausal women are 1000-1200 mg/day calcium and 800-1200 IU/day vitamin D, preferably through the diet. Supplements are indicated when dietary intake does not meet their needs.

Aim: to evaluate calcium intake in postmenopausal women who take calcium supplements, determining whether their prescription is adequate.

Material and methods: observational, descriptive, cross-sectional study in women aged 50 or over who take calcium supplements, excluding those who have not taken them for more than 6 months. Calcium intake was assessed by means of surveys using Cosman's food table, focusing on the consumption of dairy products. A uni- and bivariate description is given. Confidence intervals are calculated at 95 % and contrasts are accepted when the probability of alpha error is less than 5 % (p-value < 0.05).

Results and conclusions: from a sample of 616 women, 357 participated. The average intake of calcium ingested through the diet in postmenopausal women was 872 mg/day. Some 27 % take supplements unnecessarily, as their diet covers their calcium needs. Low adherence to supplements has been reported. About half of the women do not take or do not follow the treatment correctly. It is essential to assess dietary intake before prescribing supplements and to make patients aware of the benefits of treatment and the risks of poor adherence.

Keywords:
Calcium supplementation.
Calcium intake.
Women.
Menopause.

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INTRODUCTION

The importance of calcium intake is due to its role in the prevention of osteoporosis, considered a public health problem with an increasing social and economic burden that justifies surveillance in primary care. The specific requirements for calcium and vitamin D vary throughout life and according to the current evidence in the latest version (2022) of the *Osteoporosis Guidelines* of the Spanish Society for Bone Research and Mineral Metabolism (SEIOMM) and through consulted meta-analyses, an intake of 1000-1200 mg/day of calcium and 800-1200 IU/day of vitamin D (or equivalent) is recommended in postmenopausal women, preferably through diet (1). Treatment with these supplements would be justified whenever adequate intake is not achieved through diet, either to treat a deficiency or to reach the recommended requirements.

Currently, the presentations of calcium supplements available to us are mostly carbonate salts for oral administration, associated or not with vitamin D. Their intestinal absorption improves with food intake (2) and is reduced with the concomitant administration of proton pump inhibitors (PPIs) (3). Calcium citrate is another less prescribed salt that does not require an acidic pH for its absorption nor administration with food. Elemental calcium is the actual amount present in the supplement. Calcium carbonate contains 40 % elemental calcium, so 1250 mg represents 500 mg of elemental calcium. Citrate has 21 %. Based on the reviewed evidence, excessive amounts in calcium intake defined as greater than 2000 mg/day can be potentially harmful (6), and doses > 500 mg/day should be administered in divided doses. Higher doses are associated with a plateau in calcium absorption that may prevent a positive balance of this nutrient (2).

There are several systematic reviews suggesting that, administered in isolation, calcium supplements with or without vitamin D help reduce bone mass loss, but their impact on fracture prevention is limited or uncertain for most of the general population. The only population group with clear evidence of efficacy in fracture prevention (reducing the risk of non-vertebral fracture and, more marginally, hip fracture) is the institutionalized population older than 65 years with a high prevalence of hypovitaminosis D and low calcium intake (4). The benefit is less clear for older people living in the community and in the general healthy population, that is, without established osteoporosis, where according to the evidence of this meta-analysis, it is very difficult to justify a large-scale intervention with calcium supplements (5).

Supplements, in addition to the known adverse events mentioned in the technical data sheet, mainly GI disorders (6), are also related to the possibility of other highly controversial unwanted effects such as nephrolithiasis and cardiovascular events. To date, there is no clear evidence associating calcium supplements with an increased risk of developing kidney stones, although caution must be exercised when assessing results since the true incidence rate may be underestimated, given that a homogeneous system for assessing events has not been found, nor has it been the main variable under study. Taylor's study (7) reports that higher dietary calcium intake is independently associated with a lower risk of kidney stones; in contrast, the randomized clinical trial (8) of the Women's Health Initiative (WHI) does not confirm this relationship and found a 17 % excess in the incidence rate of kidney stones in the group that received the supplement. However, the work by Harris et al. (9) reported that adequate hydration may help reduce this risk.

The initial suggestion that triggered the debate about the probable cardiovascular risk came from a meta-analysis by Bolland et al. in 2010, which found a 27 % increase in the risk of myocardial infarction in women taking calcium supplements (10). Since then, the topic has led to multiple reviews with contradictory results. A meta-analysis by Myung et al. (11) found that the use of calcium supplements was significantly associated with a 15 % increased risk of cardiovascular disease and coronary heart disease in healthy postmenopausal women; in contrast, the meta-analyses by Chung (12) and Sim Ming Gin et al. (13) published results to the contrary, reporting that calcium supplements are not associated with any significant risk of coronary heart disease, stroke, or all-cause mortality. The controversy is not settled, as methodological deficiencies have been raised, and the discrepancies in these results remain a controversial issue, so firm conclusions on the role of supplements on cardiovascular risk are discarded.

The biological plausibility of these findings is argued by the fact that the use of calcium supplements abruptly increases circulating calcium levels and may contribute to vascular calcification and other pathophysiological processes that occur at the level of the surface of blood vessels (14), while the intake of calcium-rich foods, due to the fat and protein content they entail, leads to a slower intestinal transit that causes minor changes in serum calcium levels.

The objective of this study is to identify the dietary calcium intake of postmenopausal women who are taking supplements and to analyze whether the indication is appropriate according to the recommendations.

Hypothesis: there is a tendency to prescribe calcium and calcium/vitamin D supplements in postmenopausal women who already have an adequate dietary intake and do not present risk factors that would make them benefit from this supplementation.

MATERIAL AND METHODS

Observational, descriptive, and cross-sectional study.

Study setting: a primary care center in the Bages region (Barcelona, Spain).

Study period: a full year from January through December 2023.

Inclusion and recruitment criteria: all women older than 50 years registered at the Primary Care Center (CAP) who are taking supplements and who sign the corresponding informed consent have been included.

Exclusion criteria: women who have not gone to the pharmacy to collect their calcium supplement for more than 6 months, those who cannot understand the nature of the study, or those who do not agree to participate in the study.

Sample size: non-probabilistic or convenience sampling. The female population assigned to the CAP is 12,598 women, of whom 5,126 (40.69 %) are ≥ 50 years of age, and of these, 616 women have prescribed calcium supplements with or without vitamin D.

Population and sample calculation: for a confidence level of 95 %, from the population subgroup of 616 women with a heterogeneity of 50 %, a margin of error of 50 %, and a confidence level of 95 %, a minimum sample of 237 women is necessary.

Data collection and source of information: for each of the patients who met the inclusion criteria and who signed the written informed consent, the research team conducted an in-person or telephone survey on their dietary calcium intake. The list of dairy foods was used as the main source of calcium to determine the amount and frequency of their consumption, as detailed in table 1 of the section dedicated to the study variables.

Both the calcium intake data and the rest of the study variables were obtained through the patient survey and the review of their medical history. A questionnaire included in a Microsoft 365 Forms document, which only members of the research team had access to, was used for data collection.

Applicability: knowing the current situation of calcium consumption in postmenopausal women will allow us to introduce the necessary changes, since it is evident that reaching the recommended calcium requirements contributes to improving bone mineral density and reducing the risk of fracture (Table I).

Main variable: daily dietary calcium intake (mg/day) reported by the respondents, following the Cosman survey model, on the servings per day they take of each of the dairy products.

Secondary variables: age (years); institutionalized (yes, no); time (in months) taking supplements; professional who initiated the prescription of calcium supplements: family doctor, Rheumatology, Traumatology, Gynecology, private, others (Oncology, Internal Medicine); possible side effects related to calcium supplements (flatulence, constipation, nausea and/or vomiting, discomfort), osteoporosis or osteopenia confirmed by bone densitometry, and concomitant osteoporosis treatment (alendronate, ibandronate, risedronate, etidronic acid, denosumab, teriparatide, raloxifene, bazedoxifene).

DATA ANALYSIS

A univariate and bivariate description of the variables has been conducted.

Quantitative variables are expressed as arithmetic mean and standard deviation, and the qualitative ones as absolute and relative frequencies.

Table I. Survey model on the consumption of dairy products*

Product	Estimated mg calcium/serving	No. servings/day	Daily calcium (mg)
Milk (1 glass 200 mL)	250	-	-
Milk with calcium (1 glass 200 mL)	320	-	-
Natural whole yogurt (125 mL)	225	-	-
Yogurt with calcium	400	-	-
Custard, cream, rice pudding	120	-	-
Fresh cheese (100 g)	200	-	-
Cured cheese (2 slices or 50 g)	400	-	-
Calcium in non-dairy foods	250 mg calcium/day		
		Total daily (mg calcium)	-

**Adapted from Cosman et al. Osteoporos Int 2004.*

Bivariate contrasts have been performed with the Student's t-test or the Mann-Whitney test depending on their distribution when dealing with dichotomous qualitative variables, provided that the quantitative variable has a normal distribution.

For quantitative variables, Pearson's chi-square test with Fisher's correction has been used. The SPSS v 29.0 statistical analysis program was used for data analysis. Confidence intervals for the parameters were calculated at 95 %, and differences reaching a *p*-value < 0.05 were considered statistically significant.

CONFLICTS OF INTEREST

This project has the accreditation of the Research Ethics Committee with drugs (CEIm) of the IDIAP Jordi Gol with code 22/166-P in the session of 26/10/2022.

The authors declare that no experiments have been conducted on humans or animals for this research and that informed consent has been obtained from the patients.

No patient data appears in this article.

There are no conflicts of interest, and no funding has been received.

RESULTS

The sample consisted of a total of 357 women, and Figure 1 shows the flow of patients from the identification of candidates to their participation. The mean age of all of them was 73.0 years (SD, 10.2). Twenty-one resided in an institution (5.9 %) and their age was significantly higher (mean of 79.5 years; SD, 11.3) (Student's t-test, $p < 0.05$) (Fig. 1).

One third (33.9 %) of participants belonged to the 70 to 79 years age group, 24.6 % to the 80 to 89 years group, and 25.5 % to the 60 to 69 years group. A total of 10.9 % belonged to the 50 to 59 years group and 5.0 % to the 80 to 89 year age group. Regarding calcium consumption, participants reported a mean of 872 mg of calcium per day (SD, 325), and figure 2 presents dietary calcium consumption by age groups, where contrast (Kruskall-Walis H test) does not reach statistical significance ($p > 0.05$). Therefore, despite the absolute oscillations observed in the figure (mean consumption by age groups), consumption is not different. There is a group of 262 women (73.4 %) who take < 1000 mg and, therefore, 95 women (26.6 %) take > 1000 mg of calcium (Fig. 3).

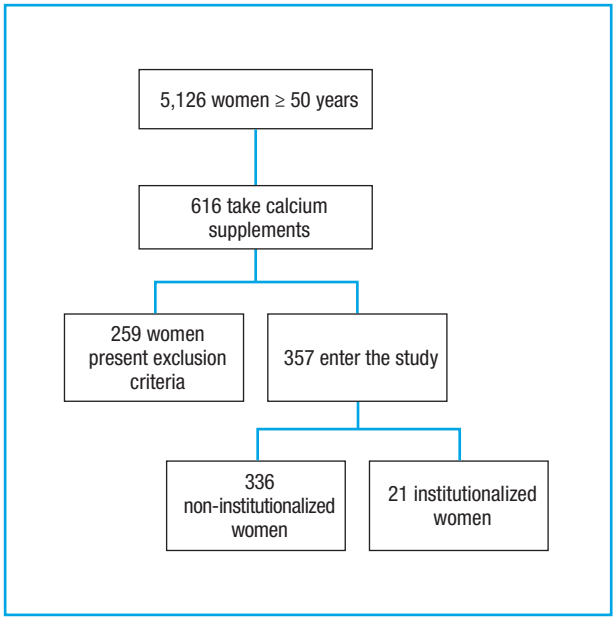

Figure 1. Flow diagram.

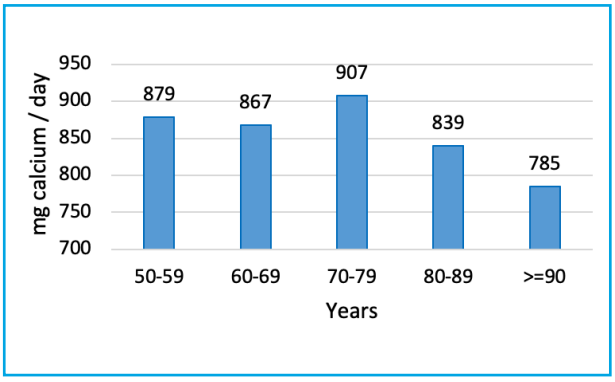

Figure 2. Mean dietary calcium consumption by age groups.

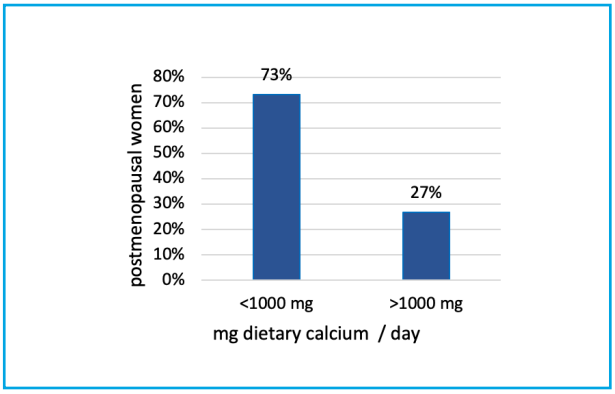

Figure 3. Dietary calcium consumption in postmenopausal women.

Regarding dairy sources of calcium (Table II), milk, consumed by 76.3 % of women, is the most consumed dairy product. Thus, it is seen that women who consume milk, milk supplemented with calcium, or natural whole yogurts have a significantly higher calcium intake (Student's t-test, $p < 0.05$) than women who do not consume these products. Furthermore, consuming other dairy specialties does not entail a higher intake vs those who do not consume these products.

A total of 26.61 % of the study participants (95) report a calcium intake > 1000 mg/day. If we break down the results by place of residence, we observe that in institutionalized women, the percentage that meets the requirements through diet is 23.8 % (5). No statistically significant differences are obtained between dietary calcium intake and place of residence (chi-square test, $p \geq 0.05$).

Regarding the side effects of diet and calcium, 10.4 % of the women included express non-specific discomfort, 8.68 % constipation, and 8.40 % flatulence (Table III). Nausea and/or vomiting is observed in 3.36 % of the women included, and these 12 women consume significantly more calcium than those who do not present them (Student's t-test, $p < 0.05$). Those who report flatulence (30 women), constipation (31), or non-specific discomfort (37) do not show significant differences in daily calcium intake.

Calcium consumption by densitometry result is presented in table IV, and no significant differences are identified in calcium consumption by densitometry result.

Table V describes that most women do not receive any concomitant treatment, and among those who do, the most prescribed (Table VI) has been alendronate at 17.1 %, followed by denosumab at 8.12 %.

It can be observed, then, that 44 % of the indicated calcium supplements are prescribed by specialists, and among them, rheumatologists stand out with 17.6 %. In the comparison to family doctors and all others, there are no significant differences in calcium intake, and the percentages of postmenopausal women who take > 1000 mg of calcium per specialist are not different (chi-square test > 0.05).

Table II. Milligrams of calcium from daily diet according to dairy source consumption

		Consumption		*p*-value
		Mean (mg/day)	SD	
Drink milk	No	675.73	329.16	*< 0.001*
	Yes	931.03	300.33	
Drink calcium-fortified milk	No	855.67	324.00	*0.003*
	Yes	1025.15	300.93	
Eat plain whole yogurt	No	751.52	350.23	*< 0.001*
	Yes	914.38	293.18	
Eat calcium-fortified plain yogurt	No	737.66	349.68	0.063
	Yes	1003.33	274.35	
Eat flan, custard, or rice pudding	No	725.94	354.86	0.121
	Yes	885.62	246.08	
Eat fresh cheese	No	699.90	431.76	0.443
	Yes	753.57	250.54	
Eat semi-cured Manchego cheese	No	716.50	473.37	0.498
	Yes	644.58	255.91	

Table III. Analysis of side effects in relation to daily calcium consumption

		Total mg calcium/day		*p*-value
		Mean	SD	
Flatulence	No	875.03	328.92	0.466
	Yes	834.33	285.64	
Constipation	No	872.43	318.97	0.892
	Yes	862.58	390.67	
Nausea and/or vomiting	No	868.04	329.72	*0.017*
	Yes	971.25	121.11	
Non-specific discomfort	No	873.47	331.24	0.709
	Yes	855.27	272.68	

Table IV. Distribution of densitometry results according to daily calcium consumption

	Up to 1000 mg/day of calcium	> 1000 mg/day of calcium
Normal or not performed	125	48
Osteopenia	44	13
Osteoporosis	93	34
Total	*262*	*95*
Chi-square test; p > 0,05 (p = 0,764).		

Table V. Distribution of concomitant anti-osteoporosis treatment

	Global sample	*n*
Concomitant anti-osteoporosis treatment		357
Alendronate	61 (17.1 %)	
Bazodoxifene	1 (0.28 %)	
None	257 (72.0 %)	
Denosumab	29 (8.12 %)	
Ibandronate	1 (0.28 %)	
Raloxifene	1 (0.28 %)	
Risedronate	2 (0.56 %)	
Romosozumab	2 (0.56 %)	
Teriparatide	3 (0.84 %)	

Table VI. Professional who initiates the prescription of calcium supplements with a medical prescription

Prescribing professional	*n*	%	≤ 1000 mg calcium/day	> 1000 mg calcium/day
Family physician	200	56.0 %	155 (77.5 %)	45 (22.5 %)
Rheumatology	63	17.6 %	45 (71.4 %)	18 (28.6 %)
Traumatology	31	8.7 %	—	—
Gynecology	11	3.1 %	62 (66 %)	32 (34 %)
Others (Oncology, Internal Med.)	48	13.4 %	—	—
Private	4	1.1 %	—	—
Total	*357*	*100 %*	*262 (73.4 %)*	*95 (26.6 %)*
Chi-square test; p > 0.05 (p = 0.105).				

DISCUSSION

Daily calcium intake has been estimated using the simplified table by Cosman et al. (15), validated as a practical and indicative tool and widely used in population studies. It is easy to use and allows a rapid estimation of calcium intake from the servings of calcium-rich foods consumed daily. When a food is consumed less frequently than once a day, it is necessary to estimate the average daily intake by dividing the calcium content by the days of the week.

Literature shows considerable variability in the results of dietary calcium intake, but the majority of the population at risk of osteoporosis and similar to our data has a deficient intake and does not reach the recommended 1000-1200 mg/day. In this regard, the ANIBES (Anthropometry, Intake and Energy Balance in Spain) epidemiological study (16) published in 2017, and conducted through surveys on nutritional habits of declared dietary intake of three days in > 2000 individuals (men and women of a very wide age range in Spain), estimated that the mean daily amounts of calcium ingested through the diet of women older than 65 years was 662 mg/day. Similarly, the study by Serra et al. (17) evaluated dietary intake through two 24-hour recalls on non-consecutive days and indicates an intake of 782.7 mg/day of calcium in Catalan women

aged 18 to 64 years. Similarly, a study by Arriaza et al. (18) conducted with 250 Spanish women between 45 and 65 years of age evaluated the declared dietary intake of three days, and only 14 % had an intake > 1000 mg/day. As a drawback, the 24-hour recall method may under- or overestimate consumption if the day in question is not representative of general habits. This is a method similar to the one we used in our study, which is based on daily servings of dairy products only.

We found results contrary to those reported in our setting in the study by Tao et al. (19), where participants were Spanish postmenopausal women with osteoporosis, and they used a telephone survey, the Spanish Food Frequency Questionnaire (FFQ), and a brief survey on calcium and vitamin D intake as methods. Possible answers included "never," "annual," "monthly," "weekly," or "daily." The mean dietary calcium intake was 1239 mg/day, generally sufficient in terms of the recommended daily intake. Bruyère et al. (20) in Spanish postmenopausal women with osteoporosis, without specifying the type of survey, reported a dietary calcium intake of 1074 mg/day, also higher than our findings. The studies in which calcium intake is below requirements are conducted in the general population; however, the studies with an intake higher than needs were conducted in postmenopausal women with osteoporosis. Probably in both studies their consumption was higher due to greater motivation.

Our results reflect that milk in all its versions was the most consumed dairy product, followed by fermented dairy products (yogurt and cheese). Current scientific evidence (21) indicates that the total intake of dairy products, both skimmed and whole, is neutrally or even beneficially associated with the risk of cardiovascular disease.

The importance of increasing the consumption of dairy products upon reaching menopause is especially evident in relation to covering the recommended guidelines; specifically, the study by Ortega-Anta (22) shows that the calcium intake in postmenopausal women who consume the 3 recommended servings of dairy products per day (as we used in our study) is significantly higher (1346 ± 310.3 mg/day) than that of postmenopausal women with lower dairy product consumption (874.1 ± 259.9 mg/day). A total of 9.52 % of women consume milk supplemented with calcium, and 5.22 % consume natural yogurts enriched with calcium. Parallel to the difficulty of achieving the recommended calcium guidelines, calcium-enriched foods are proliferating in the current market to provide more calcium to the diet, not only in dairy products but also in other nutrients (23), with serious doubts about their bioavailability (19). Following this line, and despite this variable not being included in the study, many of the patients verbalized the consumption of plant-based milks as a substitute for animal-based milk.

Lack of compliance is a fairly widespread phenomenon, and our study has also highlighted the lack of adherence of patients to taking supplements. Most patients were excluded for not collecting the supplement for more than 6 months, representing approximately one in 2 postmenopausal women, and a large number of those who entered the study verbalized erratic self-reported compliance with supplement intake. Very similar percentages to ours have been reported by other authors such as Sanfelix-Genovés et al. (24) in a publication conducted in Spain, where they estimated that compliance with taking calcium and/or vitamin D supplements was around 50 %. A systematic review (25) of the literature found an adherence rate of 67 % within the first year of treatment, with a mean persistence of 180 days/year of treatment (the review did not include any studies conducted in Spain). Similarly, the analysis of the degree of therapeutic compliance analyzed by Carbonell et al. (26) shows that according to the Haynes-Sackett self-reported compliance test, 68.7 % of patients were non-compliant, and according to the Morisky-Green test, 11.2 %. Consistent with other reviews (27), there is unanimity regarding the determining factors of poor adherence to supplements described by women. Despite this variable not being included in the study, they reported problems of tolerance, poor palatability, and lack of motivation, more frequent in polymedicated patients.

Regarding the possible side effects of supplements, there is little research that has explored them; in our work, they have been infrequent, with constipation followed by dyspeptic discomfort in the form of flatulence and malaise being the most frequent (28).

In our research, 36.4 % of women had osteoporosis confirmed by DEXA densitometry and 21 % osteopenia, percentages inverted compared to the evidence provided by the cross-sectional study of the National Health and Nutrition Examination Survey (NHANES) (29), which collected data from 4012 postmenopausal women, where the prevalence of osteoporosis determined by BMD (DEXA) reached 9.2 % and that of osteopenia 59.6 % in the 5 cycles that the study lasted from 2005 to 2018. A possible explanation for these differences is that in our study, all women were taking supplements.

Our results indicate that the calcium supplement is mostly not accompanied by any antiresorptive drugs or bone-forming agents. If prescribed, and in line with the article by Langdahl et al. (30), we observe that oral bisphosphonates, particularly alendronate, are the first-line therapy, and together with denosumab, they are the most used antiresorptive therapies.

More than half of the calcium supplements have been prescribed by the family doctor. However, the publication by Ensrud et al. (31) draws our attention, where it is noted that the treatment of osteoporosis and frac-

ture prevention strategies are often not addressed by primary care physicians, even in older patients with recent fractures, and in line with other authors (28), it calls for maximizing efforts from primary care to improve the rates of diagnosis and treatment of osteoporosis in postmenopausal women. A total of 17.64 % of supplements have been prescribed by the rheumatologist in women mostly followed in this service for osteoporosis, and despite this, no higher dietary calcium intake has been observed. A non-negligible percentage (13.4 %) have been recommended by Oncology in patients diagnosed with estrogen receptor-positive breast cancer on adjuvant therapy with aromatase inhibitors, which has been correlated with an increased risk of bone loss and fractures (the annual loss in these women after 1 year is 2.6 % at lumbar level and 1.7 % at femoral level) (32). The least prescribing services were Traumatology and Gynecology. In the latter case, the sample is small, but the study by Arriaza et al. (18) concludes that half of the gynecologists prescribe calcium prophylactically to women between 45 and 65 years of age.

The strength of this study lies in having used the simplified Cosman table as a system for evaluating calcium intake, which is easy and practical with a rapid estimation and reasonable accuracy to make the relevant recommendations. It has the advantage of adapting to our usual clinical practice and, therefore, more closely approximating the real working conditions of a family doctor.

The sample size has allowed us to have reasonable certainty regarding the confidence in the results, as well as the conclusions derived from it.

As limitations of this research, we do not have any biomarker that provides the calculation of calcium intake. Intake has been self-reported, which may lead to recall bias, errors in intrapersonal variability that may underestimate or overestimate consumption, and other subjective factors on the part of the patients.

CONCLUSIONS

After analyzing our study, it appears that the dietary calcium intake in 73 % of postmenopausal women taking supplements is insufficient (872 mg/day) and is 13 % below the recommended guidelines. We also identify 27 % of postmenopausal women who are taking supplements unnecessarily, since their diet already covers the requirements. The observation of a high rate of lack of adherence to taking calcium supplements has been highlighted.

As perspectives and proposals for improvement, these results support the need to promote greater dietary calcium intake to meet needs (an agile way is to recommend three servings of dairy products per day). To make an adequate medical prescription and avoid unnecessary supplementation, it is essential to evaluate dietary calcium consumption and only prescribe supplements if requirements are not met. On the other hand, to correct this tendency of lack of adherence, the need to explain to the patient the importance and benefits of taking supplements, as well as the risks to bone health related to inadequate follow-up, is emphasized.

KEY POINTS

- Calcium intake in postmenopausal women taking supplements is insufficient and does not reach the recommended daily dose of 1000-1200 mg/day.
- Almost a third of postmenopausal women are unnecessarily supplemented since dietary calcium intake already covers the necessary requirements.
- An agile way in primary care to increase calcium intake is to recommend 3 servings of dairy products per day.
- Adherence to supplements is very low and their compliance very erratic due to lack of motivation and side effects.
- It is necessary to evaluate dietary calcium intake before prescribing supplements, and if they are prescribed, inform patients of the benefits of taking them and the risks of poor follow-up.

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