THE ROLE OF RANKL AND FGF23 IN ASSESSING BONE TURNOVER IN TYPE 2 DIABETIC PATIENTS

G. Niță¹, O. Niță¹, A. Gherasim^{1,*}, L.I. Arhire¹, A.M Hergheliegiu², L. Mihalache¹, C. Tuchilus¹, M. Graur¹

¹“Grigore T Popa” University of Medicine and Pharmacy, Faculty of Medicine, Iasi, ²“Carol Davila” University of Medicine and Pharmacy, Faculty of Medicine, Bucharest, Romania

Abstract

Context. Type 2 diabetes is a chronic metabolic disease which affects bone. There is evidence in the literature about some serum markers that reflect the bone turnover metabolism, such as RANKL (Receptor Activator of Nuclear factor Kappa-b Ligand) and Fibroblast Growth Factor (FGF) 23.

Objective. We aimed to investigate the correlations between RANKL and FGF23 and other diabetes-related factors possibly influencing early bone turnover changes.

Subjects and Method. We conducted a cross-sectional analytical study on a group of 171 patients with type 2 diabetes, without Charcot’s arthropathy or a history of amputations, in which a complete history and anthropometric, clinical, biochemical and dietary evaluation were performed. We evaluated the serum level of RANKL and FGF 23.

Results. RANKL was significantly lower in patients with macroangiopathy (0.42 ± 0.15 pmol/L vs. 0.47 ± 0.2 pmol/L, $p=0.001$). The level of FGF23 was lower in patients with neuropathy (0.37 ± 0.36 pmol/L vs. 0.41 ± 0.17 pmol/L, $p=0.001$). We found that FGF23 increased with age, but decreased with the duration of diabetes. We also found an inverse relationship between FGF23 levels and HbA1c, triglycerides, diastolic blood pressure, total proteins, albuminemia.

Conclusions. RANKL was significantly lower in patients with macroangiopathy, and FGF 23 in patients with neuropathy. Therefore, more studies are needed to elucidate their role in early bone turnover changes.

Keywords: RANKL, FGF23, bone turnover, type 2 diabetes.

INTRODUCTION

Diabetes is a very common chronic metabolic disease, with numerous complications, among which the impact on bone health is frequently mentioned in the literature. It is a disease that affects the bone through multiple mechanisms, such as the direct effects

of insulin resistance and hyperglycemia in the bone and bone marrow, advanced protein glycation end products (AGE), abnormal cytokine production and neuro / muscular / skeletal disorders (1).

Patients with type 2 diabetes have an increased risk of fractures, despite normal or high bone mineral density (BMD), a risk attributed to poorer bone quality and changes in bone microarchitecture and local humoral environment (2).

Bone turnover is a balance between bone formation (by osteoblasts) and bone resorption (by osteoclasts). The biochemical markers used as predictors of fracture occurrence are: markers of bone formation, represented by osteocalcin, alkaline bone-specific phosphatase, alkaline phosphatase, osteoprotegerin (OPG), procollagen type 1 amino terminal propeptide (PINP) and procollagen type 1 carboxyl terminal (prop1) and markers of bone resorption, represented by N-terminal cross-linked telopeptide of type 1 collagen (NTX), C-terminal cross-linked telopeptide (CTX), tartrate-resistant acid phosphatase (TRAP), RANKL (nuclear activator receptor kappa-B ligand factor), pyridinoline (PYR), deoxypyridinoline (DPD), hydroxyproline (HP) and sclerostin (Scl) (3). RANKL and OPG are produced by the osteoblast. Sclerostin is produced by osteocytes. PINP, osteocalcin and bone-specific alkaline phosphatase are produced in the blood during bone formation. CTX, NTX and TRAP are products released into the blood during bone resorption. It is not known whether serum markers really reflect what happens in bone (3), as clinical trials including bone biopsy in patients with diabetes are extremely low.

There is a lot of discussion in the literature about the OPG / RANKL system as an important part of bone turnover metabolism. RANKL is a member of the tumor necrosis factor (TNF) superfamily, that activates osteoclastogenesis (stimulates the differentiation,

*Correspondence to: Andreea Gherasim MD, “Grigore T. Popa” University of Medicine and Pharmacy, Diabetes, Nutrition and Metabolic Diseases, Bld Independentei nr 1, Iasi, 700111, Romania, E-mail: anny_gh2005@yahoo.com

fusion, survival, activation and apoptosis of osteoclasts, leading to an increase in bone reabsorption). RANKL acts by activating a specific receptor (RANK - Receptor Activator of Nuclear factor Kappa beta) (4). RANKL binds to it, inhibiting osteoblast differentiation. OPG (RANKL's antagonist) is a marker that binds to RANKL, preventing its interaction with RANK and thus inhibiting osteoclastogenesis and preventing bone loss (5). RANK is also expressed in skeletal muscle, and activation of nuclear factor- κ B (NF- κ B) mainly inhibits myogenic differentiation, leading to dysfunction and loss of skeletal muscle (6).

Fibroblast growth factor (FGF) 23 and Klotho are hormones involved in osteo-vascular metabolism. FGF23 is a major hormone in regulating serum phosphate levels and is produced by bone in response to increased extracellular levels of phosphate and circulating vitamin D. FGF23 signalling requires co-expression of FGFR1 receptor 1 and renal α -Klotho co-receptor (7). FGF23 reduces serum phosphate levels by inhibiting both renal phosphate reabsorption and intestinal phosphate absorption by reducing serum 1,25-dihydroxyvitamin D levels (8). FGF23 inhibits the expression of ACE2 and causes the preservation of calcium and Na, actions that may have special implications in the pathophysiology of chronic kidney disease, hypertension and left ventricular hypertrophy (9). The modulators of FGF23 production are: iron deficiency, hypoxia and aldosterone (which increase FGF23 expression) and insulin signalling (which decreases FGF23 expression) (10).

Vitamin D and PTH are the main hormones that regulate bone metabolism. There is a relationship between vitamin D deficiency and diabetes and cardiovascular disease (11). In diabetes, vitamin D stops the activation of macrophages and the formation of foam cells in the vessel wall (12).

In this context, the aim of this study was to investigate the correlations between RANKL and FGF23 and other diabetes-related factors possibly influencing early bone turnover changes.

MATERIAL AND METHODS

Study population

We conducted an analytical cross-sectional study on a group of patients with type 2 diabetes, who presented during one year to our clinic for the annual check-up of complications and comorbidities. The inclusion criteria were patients with type 2 diabetes (with or without insulin treatment). The exclusion

criteria were: history of chronic kidney disease, Charcot arthropathy, diabetic foot ulcers or a history of amputations due to diabetic foot complications, other forms of autonomic neuropathy, precisely because we wanted to evaluate the early changes in bone that occur in patients with type 2 diabetes without bone disturbances. We also excluded patients with psychiatric conditions or neurologic conditions associated with cognitive impairment, active forms of cancer, diseases associated with immobilization and patients who refused to participate.

Assessment methods

The assessment of the patients included general information, anthropometric parameters, clinical evaluation, biomarkers and nutritional status assessment.

General information

We recorded the sex and age of the patients and their area of residence as being urban or rural.

Anthropometric parameters

Height was measured using a fixed stadiometer and weight was determined using a calibrated weighting scale; for both measurements patients wore only light clothes and were without shoes. We calculated body mass index (BMI) using the formula kg/m^2 and thus patients were classified as being normal weight (BMI between 18.5 kg/m^2 and 24.9 kg/m^2), overweight (BMI between 25 kg/m^2 and 29.9 kg/m^2) and with obesity ($\text{BMI} \geq 30 \text{ kg/m}^2$).

To assess abdominal obesity, Waist circumference (WC) was measured using a measuring tape at the mid-point between the lowest rib and the iliac crest, according to the recommendations of the World Health Organization and International Diabetes Federation (13). Hip circumference was also measured to allow for the calculation of the waist-to-hip ratio (WHR).

Clinical evaluation

Blood pressure was measured in all patients in dorsal decubitus after five minutes of rest, twice at both arms. Also, a complete physical examination and history taking, together with reviewing patients' charts and the current paraclinical investigations, lead to the final diagnosis of chronic complications and comorbidities: the examination of the foot assessed the presence of peripheral diabetic neuropathy, and together with ankle brachial index were used to

diagnose peripheral artery disease; cardiovascular signs and symptoms (and previous medical history) and ECG lead to a diagnosis of cardiovascular disease, fundoscopy was used to diagnose diabetic retinopathy. We assessed the presence of peripheral arterial disease separately, and macroangiopathy included previous myocardial infarction, angina pectoris or stroke.

Apart from the information regarding diabetes complications and comorbidities, we also collected data regarding the duration of diabetes, the treatment for diabetes, considering especially treatment with metformin and treatment with incretins; also, other lifestyle components: the average number of hours slept per night and current smoking status.

Biomarkers

The following biomarkers were routinely assessed as part of the annual check-up of patients: fasting glycemia, glycated haemoglobin (HbA1c), urea and creatinine (eGFR was calculated using CKD-EPI formula), ALAT and ASAT, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, uric acid, haemoglobin and haematocrit, white blood cells and platelets, C reactive protein (CRP) and fibrinogen.

As part of the nutritional assessment, we assessed the serum levels of: total proteins, albumin, calcium, magnesium, iron, vitamin D and vitamin B12.

We assessed the serum level of RANKL and FGF 23 using a 3rd generation enzyme immunoassay technique for RANKL and a multi-matrix ELISA technique for FGF23 (C-Terminal).

Nutritional status assessment

We assessed the patients' usual food and nutrient intake during the past 12 months, using the EPIC Food Frequency Questionnaire. We used this paper-based food frequency questionnaire with 130 items because it has been previously validated in Romania (14) and provides information about the habitual intake, both of nutrients (macronutrients, vitamins, minerals) and of food groups. In order to have the data mentioned previously, we introduced patients' answers to the EPIC Food Frequency Questionnaire into FETA, a software created for this use (15). Using FETA, we were able to have quantitative data (average g/day or mg/day) for all macro- and micronutrients of interest (including alcohol intake) and for eleven food groups of interest.

We assessed the usual physical activity of patients using the International Physical Activity Questionnaire (IPAQ) long form translated into

Romanian. IPAQ assesses the activity performed during the last week, dividing the opportunities for being active into four domains: at work, for transportation, at home and for spending free (leisure) time and considering three levels of intensity: walking, moderate intensity and vigorous intensity. Using the mathematical formula created for this questionnaire, IPAQ generates data regarding the average MET per week spent in each of the domains and each type of intensity.

Ethical considerations

All participants received detailed information about the nature and description of this study and were included only after they signed the informed consent for voluntary participation. The study had the approval of the Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy in Iași (No. 2324/02.02.2017).

Statistical analysis

We used SPSS Statistics version 20 for the analysis of data. For the descriptive part we used mean and standard deviation and 95% Confidence Interval for mean of scale parameters, number and percent for the ordinal ones. We analysed differences in means of scale parameters between two or more categories using One-way ANOVA (if the variables were found to be homogeneous; otherwise, we used a non-parametric test: Mann-Whitney U test for 2 samples, or the Kruskal-Wallis 1-way ANOVA for more than 2 samples). We used linear regression and Pearson correlations (or Spearman' correlations for ordinal variables) to analyse associations between variables. Statistical significance was defined at a p value <0.05.

RESULTS

We analysed a group of 171 patients, of which 83 (48.5%) were men. The mean age in the study group was 60.9 ± 10.1 years. Regarding the anthropometric indices in the studied group, we found that the average weight was 89.23 ± 17.84 kg, the mean WHR ratio was 0.98 ± 0.06 , with statistically significant differences between men and women. The mean BMI was 32.1 ± 5.4 kg/m², the mean WC was 106.03 ± 12.47 cm, without statistically significant differences between genders. We found statistically significant differences between genders for the total MET work (1375,469 for men, compared to 40,920 for women, $p=0.001$) and for the total number of minutes spent sitting down (2066.16

for men, compared to 1451.25 for women, $p=0.001$). Comparison of daily intakes by food groups by sex, showed a significantly higher average consumption of meat and meat products (145.63g compared to 112.87g in women, $p = 0.029$). The patients included in the study had a mean duration of diabetes of 7.7 ± 6.7 years. Regarding antidiabetic treatment, 73.7% were on metformin (of which 42.7% monotherapy, 19.9% in combination with other non-insulin medications, 4.7% in combination with insulin, 7.6% in combination with other non-insulin medications and insulin), 14% with incretin, 15.2% were controlled only with non-pharmacological therapy (diet).

We identified retinopathy in 11.3% and neuropathy in 36.8% of the patients. Only 2.4% of the patients had peripheral arterial disease, and 14.6% had macroangiopathy. The majority of the patients were hypertensive (74%). We found statistically significant differences between genders regarding the presence of hypertension (41.4% women, compared to 32.5% men, $p = 0.047$). The average daily calcium intake was 723.6 mg/day, and that of vitamin D was 2.3 mg/day, without statistically significant differences between the sexes.

We present in Table 1 and Table 2 the descriptive characteristics of the study population.

We analysed factors that influenced the current control of diabetes (i.e., the value of HbA1c) and the presence of chronic complications of diabetes. HbA1c was positively correlated with WC and WHR ($r^2=0.158$, $p=0.042$ and $r^2=0.215$, $p=0.008$ respectively). In our lot of patients, HbA1c was not correlated with the amount of physical exercise performed or with the usual food intake declared or with other components of lifestyle investigated. HbA1c was inversely correlated with the age of the patients ($r^2=-0.181$, $p=0.018$) but positively correlated with the duration of diabetes ($r^2=0.169$, $p=0.028$). In terms of nutritional assessment, HbA1c was inversely correlated with serum magnesium ($r^2=-0.295$, $p=0.001$) and positively correlated with serum calcium ($r^2=0.298$, $p=0.001$) and total serum protein level ($r^2=0.196$, $p=0.025$). Also, inflammation influenced current diabetes control: HbA1c was positively correlated with the level of white blood cells ($r^2=0.258$, $p=0.001$) and negatively correlated with level of fibrinogen ($r^2=-0.238$, $p=0.005$).

The presence of diabetic retinopathy was only

Table 1. Characteristics of the study population: diabetes related

Characteristic	Value
Age (years)	60.9 ± 10.1 ; 59.3-62.4 (Mean $\pm$ SD; 95 CI)
Residence (urban)	120 (70.2) (Number; %)
BMI (kg/m ²)	32.1 ± 5.4 ; 31.2-32.9 (Mean $\pm$ SD; 95 CI)
WC (cm) in men	107.1 ± 12.6 ; 104.3-109.9 (Mean $\pm$ SD; 95 CI)
WC (cm) in women	105 ± 12.2 ; 102.3-107.6 (Mean $\pm$ SD; 95 CI)
WHR	0.98 ± 0.07 ; 0.97-0.99 (Mean $\pm$ SD; 95 CI)
Systolic BP (mmHg)	142.2 ± 20.2 ; 139.1-145.2 (Mean $\pm$ SD; 95 CI)
Diastolic BP (mmHg)	84.8 ± 10.4 ; 83.2-86.4 (Mean $\pm$ SD; 95 CI)
Duration of diabetes (years)	7.7 ± 6.7 ; 6.7-8.7 (Mean $\pm$ SD; 95 CI)
Treatment with metformin	126 (73.7) (Number; %)
Incretin treatment	24 (14) (Number; %)
Diabetic retinopathy	19 (11.1) (Number; %)
Diabetic peripheral neuropathy	63 (36.8) (Number; %)
Peripheral artery disease	4 (2.3) (Number; %)
Macroangiopathy	25 (14.6) (Number; %)
Hypertension	125 (73.1) (Number; %)
Dyslipidemia	106 (62) (Number; %)
Fasting plasma glucose (mg/dL)	152.2 ± 48.5 ; 144.8-159.5 (Mean $\pm$ SD; 95 CI)
HbA1c (%)	7.2 ± 1.3 ; 6.9-7.3 (Mean $\pm$ SD; 95 CI)
Total cholesterol (mg/dL)	204.6 ± 50.6 ; 196.9-212.3 (Mean $\pm$ SD; 95 CI)
HDL-cholesterol (mg/dL)	47.8 ± 13.2 ; 45.6-49.9 (Mean $\pm$ SD; 95 CI)
LDL-cholesterol (mg/dL)	131.1 ± 45.9 ; 123.5-138.6 (Mean $\pm$ SD; 95 CI)
Triglycerides (mg/dL)	179.3 ± 84.2 ; 145.8-181.9 (Mean $\pm$ SD; 95 CI)
Uric acid (mg/dL)	5.5 ± 1.4 ; 5.3-5.7 (Mean $\pm$ SD; 95 CI)
e GFR (mL/min./1.73 m ²)	85.2 ± 16.4 ; 82.7-87.7 (Mean $\pm$ SD; 95 CI)
ALAT (U/L)	31.5 ± 26.5 ; 27.4-35.6 (Mean $\pm$ SD; 95 CI)
ASAT (U/L)	24.1 ± 17.6 ; 21.4-26.8 (Mean $\pm$ SD; 95 CI)

SD=standard deviation, CI=Confidence Interval for mean, BMI=body mass index, WC=waist circumference, WHR=waist-to-hip ratio, BP=blood pressure, e GFR=estimated glomerular filtration.

associated with a significantly higher value of HbA1c ($8.35 \pm 1.65\%$ vs. $7 \pm 1.1\%$, $p < 0.001$). The presence of neuropathy was significantly associated with older age (64.2 ± 8.7 years vs. 58.9 ± 10.4 years, $p = 0.001$), less hours of sleep per night (6 ± 1 hours/night vs. 6.6 ± 1.2 hours/night, $p = 0.048$) and the following biological parameters: lower serum iron (82.9 ± 26.4 mg/dL vs. 96 ± 32 mg/dL, $p = 0.02$), lower haemoglobin (13.5 ± 1.33 g/dL vs. 14 ± 1.26 , $p = 0.02$) and lower eGFR (79.7 ± 17 mL/min./1.73 m² vs. 88.4 ± 15.1 mL/min./1.73 m², $p = 0.001$). In patients with peripheral artery disease, white blood cells and percent neutrophils were significantly higher than in patients without this chronic complication (10.06 ± 1.16 *1000/mm³ vs. 7.46 ± 1.94 *1000/mm³, $p = 0.023$; $68.8 \pm 2.45\%$ vs. $59.2 \pm 7.37\%$, $p = 0.026$, respectively). Patients with macroangiopathic complications were found to spend significantly more minutes per week sitting

(1842.2 ± 965.7 min./week vs. 1085.8 ± 964.6 min./week, $p = 0.012$) and to declare a significantly higher regular intake of proteins (101.2 ± 74 g/day vs. 80.2 ± 29 g/day, $p = 0.049$) and meat and derivatives (170.8 ± 139 g/day vs. 123.6 ± 70.5 g/day, $p = 0.043$).

We further evaluated the factors which influenced the level of RANKL and we present only the associations which reached statistical significance. We found that RANKL was inversely correlated with WHR ($r^2 = -0.194$, $p = 0.019$) and the amount of alcohol consumed on average ($r^2 = -0.205$, $p = 0.033$) and was positively correlated with the usual intake of nuts and seeds ($r^2 = 0.201$, $p = 0.036$) and fish ($r^2 = 0.223$, $p = 0.02$). The only association between RANKL and the chronic complications was with the macroangiopathic complications, RANKL being significantly lower in patients with macroangiopathic complications

Table 2. Characteristics of the study population: nutritional status related

Characteristic	Value
Sleeping duration (hours/night)	6.44 ± 1.18 ; 6.21-6.67 (Mean $\pm$ SD; 95 CI)
Smokers	13 (7.6) (Number; %)
Alcohol intake (g/day)	6.24 ± 9.29 ; 4.56-7.93 (Mean $\pm$ SD; 95 CI)
Total MET Work (METS/week)	159.9-1167.5 (95 CI)
Total MET Transport (METS/week)	667.2-1083.9 (95 CI)
Total MET Household (METS/week)	1572.8-2752.5 (95 CI)
Total MET Leisure (METS/week)	165.7-393.1 (95 CI)
Total MET Walking (METS/week)	935-1539.9 (95 CI)
Total MET Moderate (METS/week)	1674.9-2889.8 (95 CI)
Total MET Vigorous (METS/week)	0.02-17.45 (95 CI)
Total METS per week	2800-4257.1 (95 CI)
Sitting time (minutes/week)	1544.5-1932 (95 CI)
Energy intake (kcal/day)	1705.5 ± 673.3 ; 1583.3-1827.7 (Mean $\pm$ SD; 95 CI)
Carbohydrate intake (g/day)	206 ± 88.9 ; 189.8-222.1 (Mean $\pm$ SD; 95 CI)
Protein intake (g/day)	82.2 ± 37.2 ; 75.5-89 (Mean $\pm$ SD; 95 CI)
Total lipid intake (g/day)	60.1 ± 28.1 ; 55-65.1 (Mean $\pm$ SD; 95 CI)
Cereal intake (g/day)	231.7 ± 137.3 ; 206.7-256.6 (Mean $\pm$ SD; 95 CI)
Vegetable intake (g/day)	295.2 ± 167.2 ; 264.8-325.5 (Mean $\pm$ SD; 95 CI)
Fruit intake (g/day)	277.2 ± 157.9 ; 248.5-305.9 (Mean $\pm$ SD; 95 CI)
Meat and derivatives (g/day)	128.3 ± 81.9 ; 113.4-143.2 (Mean $\pm$ SD; 95 CI)
Fish and derivatives (g/day)	33 ± 26.1 ; 28.2-37.7 (Mean $\pm$ SD; 95 CI)
Dairy intake (g/day)	238.2 ± 147 ; 211.5-264.8 (Mean $\pm$ SD; 95 CI)
Total serum proteins (g/dL)	7.43 ± 0.41 ; 7.36-7.5 (Mean $\pm$ SD; 95 CI)
Albuminemia (g/dL)	4.61 ± 0.29 ; 4.56-4.66 (Mean $\pm$ SD; 95 CI)
Serum vitamin D (ng/mL)	17.2 ± 9.5 ; 15.5-19.1 (Mean $\pm$ SD; 95 CI)
Serum vitamin B12 (pg/mL)	380 ± 198.4 ; 343.9-416.2 (Mean $\pm$ SD; 95 CI)
Serum magnesium (mg/dL)	1.95 ± 0.24 ; 1.9-1.99 (Mean $\pm$ SD; 95 CI)
Serum total calcium (mg/dL)	9.69 ± 0.44 ; 9.61-9.76 (Mean $\pm$ SD; 95 CI)
Serum iron (mg/dL)	92.6 ± 30.9 ; 87.2-97.9 (Mean $\pm$ SD; 95 CI)
CRP (mg/dL)	0.36-0.53 (95 CI)
Fibrinogen (mg/dL)	392.7 ± 55 ; 383.4-402 (Mean $\pm$ SD; 95 CI)
Hemoglobin (g/dL)	13.8 ± 1.3 ; 13.7-14.1 (Mean $\pm$ SD; 95 CI)
WBC (*1000/mm ³)	7.51 ± 1.96 ; 7.18-7.82 (Mean $\pm$ SD; 95 CI)
% Neutrophils	59.6 ± 7.5 ; 58.4-60.8 (Mean $\pm$ SD; 95 CI)
Platelets (*1000/mm ³)	232.7 ± 58.2 ; 222.8-242.7 (Mean $\pm$ SD; 95 CI)

SD=standard deviation, CI=Confidence Interval for mean, CRP=C reactive protein, WBC=white blood cells.

(0.42 ± 0.15 pmol/L vs. 0.47 ± 0.2 pmol/L, $p=0.001$). RANKL was also inversely correlated with the level of ALAT and ASAT ($r^2=-0.231$, $p=0.004$, $r^2=-0.203$, $p=0.012$ respectively).

We also analysed the factors with significant associations with FGF23. We found that FGF23 increased with age ($r^2=0.198$, $p=0.012$), but decreased with the duration of diabetes ($r^2=-0.177$, $p=0.025$). There was a negative correlation between FGF23 and diastolic blood pressure values ($r^2=-0.186$, $p=0.02$). FGF23 was also inversely correlated with the value of HbA1c ($r^2=-0.231$, $p=0.003$) and was lower in those with poor glycaemic control (0.36 ± 0.21 pmol/L vs. 0.43 ± 0.28 pmol/L, $p=0.041$). We also found negative correlations between FGF23 and triglycerides ($r^2=-0.171$, $p=0.03$), eGFR ($r^2=-0.219$, $p=0.006$), haemoglobin ($r^2=-0.174$, $p=0.038$), total proteins ($r^2=-0.217$, $p=0.016$) and albumin level ($r^2=-0.274$, $p=0.002$). We divided the study group into two groups, depending on the presence or absence of neuropathy. Analyzing the comparative markers of bone turnover between these groups, we found that patients with neuropathy had lower serum levels of RANKL (0.41 ± 0.14 pmol/L vs. 0.47 ± 0.20 , $p=0.066$) and FGF23, compared to those without neuropathy, with statistical significance only for FGF23 (0.37 ± 0.36 pmol/L vs. 0.41 ± 0.17 pmol/L, $p=0.001$).

When analyzing the correlations between the average daily mineral intake and the values of RANKL and FGF23, we did not find statistically significant associations. There were no significant correlations between the average daily intake of vitamins and the values of RANKL and FGF23. We did not find any statistical significant correlation between the levels of FGF 23 or RANKL and blood vitamin D and calcium levels.

DISCUSSION

In our group of patients, we did not find a correlation between RANKL and the duration of the disease or control of diabetes (value of HbA1c) and other metabolic parameters. However, RANKL was lower in patients with macrovascular complications, in patients with a higher degree of abdominal obesity or hepatic liver enzymes (possibly in the context of hepatic steatosis) and also in those patients who drank more alcohol and ate less nuts, seeds and fish. In the literature, the OPG/RANK/RANKL system is associated with the regulation of glucose homeostasis. Hyperglycemia is an inhibitor of RANKL, inhibits

the differentiation and function of RANKL-mediated osteoclasts, and suppresses osteoclastogenesis (16). Blocking this pathway has been shown to improve hepatic insulin resistance and prevent the development of diabetes (17).

The duration of diabetes seems to negatively affect bone metabolism, but also poor glycemic control (HbA1c $\geq 7.5\%$) has been shown to be associated with an increased risk of fracture (18). Some authors have reported low levels of RANKL in diabetic patients compared to normal subjects (19). In our group of patients with type 2 diabetes we did not find a correlation between the RANKL level and the duration of the disease or the level of glycaemic control (assessed by HbA1c). The decrease in RANKL observed in literature was attributed to immature osteoblasts and an increase in the number of osteoclasts (20). Some authors also found that serum RANKL levels were lower and OPGs were higher in diabetic patients compared to nondiabetics and prediabetics (21). The highest serum concentration of RANKL was associated with a significantly increased risk of developing type 2 diabetes (22). Other authors did not find significant differences in RANKL level between patients with diabetes and the control group, nor between type 1 and type 2 diabetes. The authors showed that plasma blood glucose had a significant effect of modifying RANKL, but not HbA1c, nor the duration of diabetes (23).

Serum levels of FGF-23 have been shown to be elevated in patients with type 2 diabetes, giving them an increased risk of developing cardiovascular disease (24). However, the results are controversial in the literature. Thus, in some studies, diabetes was correlated with higher circulatory FGF23 levels (24), while other studies did not find these correlations (25). Our results show that FGF23 increases with age but decreases with the duration of diabetes and is inversely correlated with HbA1c.

Our results showed no association between RANKL level and the presence of neuropathy, but RANKL was significantly decreased in patients with macroangiopathy. In the same way, Bourron *et al.* conducted a cross-sectional study in 198 patients with type 2 diabetes and high cardiovascular risk, in which they investigated the relationship between neuropathy and RANKL and OPG levels, but found no association between them (26).

It seems that RANKL plays a central role in accelerating the process of arterial calcification (27), which is more common in diabetics with peripheral neuropathy, with osteolysis (28). Elevated serum levels

of FGF23 have been associated with an increased risk of complications (29). Contrary to these results, our study showed lower level of FGF23 in patients with neuropathy. Also, our results showed no associations between FGF23 and the average intake of phosphate or other nutrients of interest. A high-fiber diet is associated with low levels of FGF23, because it means a low consumption of animal protein and therefore a low intake of phosphorus (30). It has been found that high calcium intake is also associated with elevated levels of FGF23 (31), on the same principle as consuming foods of animal origin, also rich in phosphorus (32). Adequate intake of calcium and vitamin D is essential for bone health, prevention of osteoporosis and fractures, and for cardiovascular health (32). Although we expected to find some associations between the daily intake or the plasma levels of vitamin D and calcium, and the levels of RANKL and FGF23, as surrogate markers of bone disturbances, we did not find any. Other studies reported no correlation with plasma vitamin D (31), but a positive association between calcium levels (33) and calcium intake and higher FGF23 (31).

Our results showed no association between FGF23 and physical exercise, contrary to the results of recent study, albeit on mice: this study showed that chronic exercise (60 minutes a day for a week) not only increases serum FGF23 levels but also regulates FGF23 mRNA expression (34). In our group of patients with type 2 diabetes, no relationship was found between FGF23 levels and anthropometric parameters. A cross-sectional analysis in the EPIC-Germany study revealed that an elevated level of FGF23 was positively correlated with abdominal circumference and BMI in women compared to men. Regarding the diet, the participants in the group with increased FGF23 had a higher caloric intake, higher intakes of phosphorus, calcium, as well as a higher phosphorus / protein ratio. The authors show that male gender and iron intake are negatively correlated with higher concentrations of FGF23, and calcium and energy intake are positively correlated with higher concentrations of FGF23 (31).

Research shows that higher FGF23 concentrations have been associated with obesity and insulin resistance (35). Some authors concluded in their research on a group of men with normal renal function, that older age, BMI, phosphorus intake, smoking status, hypertension, higher levels of PTH, phosphate, triglycerides, uric acid and biomarkers of inflammation have been independently associated with higher plasma concentrations of FGF23 (36). However, our results do not confirm these data, in our group there is an inverse

relationship between FGF23 levels and metabolic parameters represented by HbA1c, triglycerides, diastolic blood pressure. We observed the same inverse relationship between the value of FGF23 and the protein status (total proteins, albuminemia); although a more specific nutritional biomarker would have been prealbumin (37), which we did not measure.

It should be mentioned that in the studied group there were some patients with peripheral neuropathy, but no patients with advanced stages of the disease (autonomic neuropathy) or Charcot's neuroarthropathy and most of the data in the literature is reported especially for these categories of patients. Patients with Charcot neuroarthropathy had higher levels of RANKL *versus* patients without neuropathy and *versus* control subjects (38). Abnormal RANKL levels may be the basis for osteolysis and arteriolar calcification seen in patients with Charcot's neuroarthropathy.

In the study conducted by Donate-Correa *et al.* (29) FGF23 concentration was significantly higher in patients with diabetic foot (23.8 (17-32.2) vs. 15.5 (10.1-24.5) pg/mL, $p < 0.01$), showing an increase of 30.3%, compared with patients without diabetic foot. In our group, there were no patients with diabetic foot.

Also, in our group of patients, we found a better glycaemic control and a shorter duration of diabetes, than that which is usually mentioned in literature. In relation to the findings about RANKL and FGF23: we found an average HbA1c of $7.1 \pm 1.3\%$, compared to an average $8.01 \pm 1.9\%$ in literature and an average duration of diabetes of 7.7 ± 6.7 years compared to 13.3 ± 7.6 years in the literature (39).

We found no association between the level of RANKL and incretin medication (the number of patients treated with these drugs was however small) or treatment with metformin. Some authors suggest that sitagliptin suppressed RANKL differentiated from osteoclasts and bone resorption. Subsequent studies have shown that sitagliptin inhibits osteoclastogenesis (39). Other authors note that metformin decreases RANKL activity in bone and may also decrease RANKL activity in the liver, the mechanisms of which are unknown (22).

We conducted an analytical cross-sectional study in which we enrolled consecutively all patients which met the inclusion criteria and did not have any of the exclusion criteria. Therefore, the population studied had different characteristics in regard to the duration or control of the disease, treatment or complications. However, as we aimed to investigate the possible role of RANKL and FGF23 in identifying patients

with early bone changes, we excluded patients with Charcot arthropathy or history of amputations. This might explain the differences between our results and some of the literature data, because these parameters of bone turnover usually change in situations associated with increased bone turnover, i.e. diabetic foot or Charcot's foot, especially when acute. In fact, studies that follow these patients see the dynamics of change in their plasma level compared to the acute moment: RANKL is high at the acute phase and then decreases as it stabilizes (40).

There are several limitations in this study: the study has no control group, either of patients without diabetes, or of patients with advanced diabetic foot disease. One way to assess bone disturbances or bone quality is by estimating bone mineral density (BMD) by dual X-ray absorptiometry (DXA) and measuring TBS (trabecular bone score), the latter being especially used for estimating fracture risk. A limitation of our study is that we did not perform DXA in our patients, thus we could not investigate associations between osteoporosis and bone markers measured in our study. In our research, the patients did not benefit from more biochemical or imagistic explorations specific to test the bone status in order to better understand the associations between metabolic control and bone turnover. Another limitation of the study is that it is a cross-sectional study, and probably more information will gather when assessing these patients another time in the future.

In conclusion, in our study we did not find statistical significant correlations between serum RANKL and FGF23 and other parameters that may show early bone turnover changes and this may be due to the fact that the group of subjects was a homogeneous type 2 diabetic patients group, with a relatively short duration of diabetes, with a relatively good glycemic control, and without any history of foot complications. Further studies are required, in patients with or without diabetic foot problems, or these patients followed-up in the future, to find if indeed these bone markers have a role in estimating the risk of diabetic foot.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Starup-Linde J, Vestergaard P. Management of endocrine disease: Diabetes and osteoporosis: cause for concern?. *Eur J Endocrinol*. 2015;173(3):R93-R99.
- Poiana C, Capatina C. Osteoporosis and Fracture Risk in Patients with Type 2 Diabetes Mellitus. *Acta Endo (Buc)*. 2019;15(2):231-236.
- Starup-Linde J, Vestergaard P. Biochemical bone turnover markers in diabetes mellitus - A systematic review. *Bone*. 2016;82:69-78.
- Horowitz M, Xi Y, Wilson K, Kacena M. Control of osteoclastogenesis and bone resorption by members of the TNF family of receptors and ligands. *Cytokine Growth Factor Rev*. 2001;12(1):9-18.
- Ominsky MS, Li X, Asuncion FJ, Barrero M, Warmington KS, Dwyer D, Stolina M, Geng Z, Grisanti M, Tan HL, Corbin T, McCabe J, Simonet WS, Ke HZ, Kostenuik PJ. RANKL inhibition with osteoprotegerin increases bone strength by improving cortical and trabecular bone architecture in ovariectomized rats. *J Bone Miner Res*. 2008;23(5):672-682.
- Lee D, Goldberg AL. Muscle Wasting in Fasting Requires Activation of NF-kappaB and Inhibition of AKT/Mechanistic Target of Rapamycin (mTOR) by the Protein Acetylase, GCN5. *J Biol Chem*. 2015;290(51):30269-30279.
- Erben RG. Pleiotropic Actions of FGF23. *Toxicol Pathol*. 2017;45(7):904-910.
- Takashi Y, Fukumoto S. Phosphate-sensing and regulatory mechanism of FGF23 production. *J Endocrinol Invest*. 2020;43(7):877-883.
- Han X, Quarles LD. Multiple faces of fibroblast growth factor-23. *Curr Opin Nephrol Hypertens*. 2016;25(4):333-342.
- Takashi Y, Fukumoto S. FGF23 beyond Phosphotropic Hormone. *Trends Endocrinol Metab*. 2018;29(11):755-767.
- Maddaloni E, Cavallari I, Napoli N, Conte C. Vitamin D and Diabetes Mellitus. *Front Horm Res*. 2018;50:161-176.
- Oh J, Weng S, Felton SK, Bhandare S, Rick A, Butler B, Proctor BM, Petty M, Chen Z, Schechtman KB, Barnal-Mizrachi L, Barnal-Mizrachi C. 1,25(OH)₂ Vitamin D inhibits foam cell formation and suppresses macrophage cholesterol uptake in patients with type 2 diabetes mellitus. *Circulation*. 2009;120(8):687-698.
- International Diabetes Federation. IDF Diabetes Atlas, 9th edn. Brussels, Belgium: 2019. (Accessed April 18, 2021, at site <https://www.diabetesatlas.org>).
- Gherasim A, Arhire LI, Niță O, Străteanu R, Oprescu AC, Graur M, Mihalache L. Can the EPIC food frequency questionnaire be applied to the population in Romania?. *Rev Med Chir Soc Med Nat*. 2015;119(3):856-863.
- Mulligan AA, Luben RN, Bhaniani A, Parry-Smith DJ, O'Connor L, Khawaja AP, Forouhi NG, Khaw KT, EPIC-Norfolk FFQ Study. A new tool for converting food frequency questionnaire data into nutrient and food group values: FETA research methods and availability. *BMJ Open*. 2014;4(3):e004503.
- Wittrant Y, Gorin Y, Woodruff K, Horn D, Abboud HE, Mohan S, Abboud-Werner SL. High d(+)glucose concentration inhibits RANKL-induced osteoclastogenesis. *Bone*. 2008;42(6):1122-1130.
- Duan P, Yang M, Wei M, Liu J, Tu P. Serum Osteoprotegerin Is a Potential Biomarker of Insulin Resistance in Chinese Postmenopausal Women with Prediabetes and Type 2 Diabetes. *Int J Endocrinol*. 2017;2017:8724869.
- Dede AD, Tournis S, Dontas I, Trovas G. Type 2 diabetes mellitus and fracture risk. *Metabolism*. 2014;63(12):1480-1490.
- Secchiero P, Corallini F, Pandolfi A, Consoli A, Candido R, Fabris B, Celeghini C, Capitani S, Zauli G. An increased osteoprotegerin serum release characterizes the early onset of diabetes mellitus and may contribute to endothelial cell dysfunction. *Am J Pathol*. 2006;169(6):2236-2244.
- Sassi F, Buondonno I, Luppi C, Spertino E, Stratta E, Di Stefano M, Ravazzoli M, Isaia G, Trento M, Passera P, Porta M, Isaia GC, D'Amelio P. Type 2 diabetes affects bone cells precursors and bone turnover. *BMC Endocr Disord*. 2018;18(1):55.
- Panezai J, Altamash M, Engstrm PE, Larsson A. Association of Glycated Proteins with Inflammatory Proteins and Periodontal

- Disease Parameters. *J Diabetes Res.* 2020;2020:6450742.
22. Kiechl S, Wittmann J, Giaccari A, Knoflach M, Willeit P, Bozec A, Moschen AR, Muscogiuri G, Soric GP, Kireva T, Summerer M, Wirtz S, Luther J, Mielenz D, Billmeier U, Egger G, Mayr A, Oberhollenzer F, Kronenberg F, Orthofer M, Penninger JM, Meigs JB, Bonora E, Tilg H, Willeit J, Schett G. Blockade of receptor activator of nuclear factor-kappaB (RANKL) signaling improves hepatic insulin resistance and prevents development of diabetes mellitus. *Nat Med.* 2013;19(3):358-363.
23. Hygum K, Starup-Linde J, Harslof T, Vestergaard P, Langdahl BL. Mechanisms in Endocrinology: Diabetes mellitus, a state of low bone turnover - a systematic review and meta-analysis. *Eur J Endocrinol.* 2017;176(3):R137-R57.
24. Hu X, Ma X, Luo Y, Xu Y, Xiong Q, Pan X, Bao Y, Jia W. Elevation in fibroblast growth factor 23 and its value for identifying subclinical atherosclerosis in first-degree relatives of patients with diabetes. *Sci Rep.* 2016;6:34696.
25. Reyes-Garcia R, Garcia-Martin A, Garcia-Fontana B, Morales-Santana S, Rozas-Moreno P, Munoz-Torres M. FGF23 in type 2 diabetic patients: relationship with bone metabolism and vascular disease. *Diabetes Care.* 2014;37(5):e89-e90.
26. Bourron O, Aubert CE, Liabeuf S, Cluzel P, Lajat-Kiss F, Dadon M, Komajda M, Mentaverri R, Brazier M, Pierucci A, Morel F, Jacqueminet S, Massy ZA, Hartemann A. Below-knee arterial calcification in type 2 diabetes: association with receptor activator of nuclear factor kappaB ligand, osteoprotegerin, and neuropathy. *J Clin Endocrinol Metab.* 2014;99(11):4250-4258.
27. Bourron O, Phan F, Diallo MH, Hajage D, Aubert CE, Carlier A, Salem JE, Funck-Brentano C, Kemel S, Cluzel P, Redheuil A, Davaine JM, Massy Z, Mentaverri R, Bonnefont-Rousselot D, Gillery P, Jaisson S, Vermeer C, Lacorte JM, Bouziri N, Laroche S, Amouyal C, Hartemann A. Circulating Receptor Activator of Nuclear Factor kB Ligand and triglycerides are associated with progression of lower limb arterial calcification in type 2 diabetes: a prospective, observational cohort study. *Cardiovasc Diabetol.* 2020;19(1):140.
28. Kucera T, Shaikh HH, Sponer P. Charcot Neuropathic Arthropathy of the Foot: A Literature Review and Single-Center Experience. *J Diabetes Res.* 2016;2016:3207043.
29. Donate-Correa J, Martín-Núñez E, Ferri C, Hernández-Carballo C, Tagua VG, Delgado-Molinos A, López-Castillo Á, Rodríguez-Ramos S, Cerro-López P, López-Tarruella VC, Arévalo-González MA, Pérez-Delgado N, Mora-Fernández C, Navarro-González JF. FGF23 and Klotho Levels are Independently Associated with Diabetic Foot Syndrome in Type 2 Diabetes Mellitus. *J Clin Med.* 2019;8(4).
30. Kosk D, Kramer H, Luke A, Camacho P, Bovet P, Rhule JP, Forrester T, Wolf M, Sempos C, Melamed ML, Dugas LR, Cooper R, Durazo-Arvizu R. Dietary factors and fibroblast growth factor-23 levels in young adults with African ancestry. *J Bone Miner Metab.* 2017;35(6):666-674.
31. di Giuseppe R, Kuhn T, Hirche F, Buijsse B, Dierkes J, Fritsche A, Kaaks R, Boeing H, Stangl GI, Weikert C. Potential Predictors of Plasma Fibroblast Growth Factor 23 Concentrations: Cross-Sectional Analysis in the EPIC-Germany Study. *PLoS One.* 2015;10(7):e0133580.
32. Pieralice S, Vigeveno F, Del Toro R, Napoli N, Maddaloni E. Lifestyle Management of Diabetes: Implications for the Bone-Vascular Axis. *Curr Diab Rep.* 2018;18(10):84.
33. Parker BD, Schurgers LJ, Brandenburg VM, Christenson RH, Vermeer C, Ketteler M, Shlipak MG, Whooley MA, Ix JH. The associations of fibroblast growth factor 23 and uncarboxylated matrix Gla protein with mortality in coronary artery disease: the Heart and Soul Study. *Ann Intern Med.* 2010;152(10):640-648.
34. Li DJ, Fu H, Zhao T, Ni M, Shen FM. Exercise-stimulated FGF23 promotes exercise performance via controlling the excess reactive oxygen species production and enhancing mitochondrial function in skeletal muscle. *Metabolism.* 2016;65(5):747-756.
35. Hanks LJ, Casazza K, Judd SE, Jenny NS, Gutierrez OM. Associations of fibroblast growth factor-23 with markers of inflammation, insulin resistance and obesity in adults. *PLoS One.* 2015;10(3):e0122885.
36. Gutierrez OM, Wolf M, Taylor EN. Fibroblast growth factor 23, cardiovascular disease risk factors, and phosphorus intake in the health professionals follow-up study. *Clin J Am Soc Nephrol.* 2011;6(12):2871-2878.
37. Trufa DI, Arhire LI, Grigorescu C, Mihalache L, Nita O, Graur M, Mihai BM. Assessment of preoperative and postoperative prealbumin in thoracic surgery – a two months experience in a Romanian university hospital. *Rev Romana Med Lab.* 2015;23(1):75-86.
38. Ndiip A, Williams A, Jude EB, Serracino-Inglott F, Richardson S, Smyth JV, Boulton AJ, Alexander MY. The RANKL/RANK/OPG signaling pathway mediates medial arterial calcification in diabetic Charcot neuroarthropathy. *Diabetes.* 2011;60(8):2187-2196.
39. Reyes-García R, Rozas-Moreno P, López-Gallardo G, García-Martín A, Varsavsky M, Avilés-Perez MD, Muñoz-Torres M. Serum levels of bone resorption markers are decreased in patients with type 2 diabetes. *Acta Diabetol.* 2013;50(1):47-52.
40. Jansen RB, Christensen TM, Bülow J, Rørdam L, Holstein PE, Jørgensen NR, Svendsen OL. Bone mineral density and markers of bone turnover and inflammation in diabetes patients with or without a Charcot foot: An 8.5-year prospective case-control study. *J Diabetes Complications.* 2018;32(2):164-170.