Relationship between endothelial dysfunction and osteoprotegerin, vitamin D, and bone mineral density in patients with rheumatoid arthritis

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Abstract

Objective

We aimed to investigate whether the abnormalities in bone mineral density (BMD) that occur in patients with rheumatoid arthritis (RA) are associated with the presence of endothelial dysfunction.

Methods

Cross-sectional study encompassing 216 subjects (111 patients with RA and 105 age- and sex-matched controls) without history of cardiovascular disease. Endothelial function was determined by brachial artery flow-mediated dilatation (FMD) and BMD by dual x-ray absorptiometry (DXA) measurements. Plasma vitamin D and osteoprotegerin serum (OPG) levels were assessed in patients and controls. Multiple regression analysis was performed to study the relationship between BMD with endothelial function, taking into account vitamin D and OPG levels.

Results

After adjusting for traditional cardiovascular risk factors, vitamin D and OPG levels, BMD emerged as an independent factor associated with lower FMD values in controls, but not in patients with RA. Although OPG levels were inversely associated with FMD values in both RA patients and controls after adjusting for BMD, vitamin D showed this relationship only in the controls.

Conclusion

Whilst OPG is associated with endothelial function in RA patients and controls, vitamin D levels and BMD are related to endothelial function in controls but not in patients with RA.

Key words

rheumatoid arthritis, bone mineral density, endothelial function, cardiovascular disease, osteoprotegerin, vitamin D

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Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune disease of unknown aetiology that causes the destruction of joint cartilage. All-cause mortality is two to five times higher in RA patients than in the general population and the most frequent cause of death in RA is cardiovascular disease (1, 2). This increased rate of cardiovascular mortality in RA cannot be fully explained by the classic atherosclerosis risk factors, and it is thought to be the result of accelerated atherogenesis due to chronic inflammation (3). However, the underlying mechanisms leading to cardiovascular disease in RA are still being investigated (4, 5).

Patients with RA experience a generalised bone loss as the result of a systemic bone resorption that exceeds bone formation. Bone loss in RA may be systemic, periarticular, or focal. This is caused, in general, by immobility, cytokine-mediated systemic inflammation (*e.g.* tumour necrosis factor and interleukin-1), or by the effects of glucocorticoid therapy (6). Studies have shown that patients with RA have a 30% increased risk of major osteoporotic fracture and a 40% increased risk of hip fracture (7).

Atherosclerosis and osteoporosis appear to be epidemiologically and age-independently correlated in the general population (8). Patients with osteoporosis have higher levels of vascular calcification than those with normal bone mineral density (BMD) (9, 10) and clinical evidence has shown that osteoporosis is independently associated with cardiovascular events (11, 12) and increased cardiovascular mortality (13). Moreover, vascular calcification has been found to be related to an increased risk of fracture (14). Major advances in the understanding of the pathophysiology underlying osteoporosis and its link to vascular damage indicate that these two processes could share a common pathogenesis involving bone morphogenetic proteins such as vitamin D and the receptor activator of the RANKL-RANK-OPG pathway (receptor activator of nuclear factor κB ligand-receptor activator of nuclear factor κB - osteoprotegerin).

Osteoprotegerin (OPG) is a member of the tumour necrosis factor-related family that binds to RANKL, preventing its interaction with RANK and inhibiting osteoclast differentiation. There is now emerging evidence that OPG participates in the pathogenesis of atherosclerosis and cardiovascular diseases by amplifying the adverse effects of inflammation and several traditional risk factors such as hyperlipidaemia, endothelial dysfunction, diabetes mellitus, and hypertension (15, 16). Some epidemiological studies have also shown a positive association between OPG levels and cardiovascular morbidity and mortality (15, 17). Recent studies from our group have found an association between severe subclinical atherosclerosis and OPG levels in patients who lack clinically evident cardiovascular disease and who were undergoing anti-TNF therapy because of severe disease (18). Interestingly, the association of OPG with both carotid intima-media wall thickness and the presence of carotid plaques was independent of conventional risk factors and C-reactive protein (CRP) concentrations or disease activity (18). In patients with severe RA OPG concentrations were also associated with intercellular adhesion molecule (ICAM)-1, a biomarker of endothelial cell activation (18). More, recently, in a different cohort of unselected RA patients, we observed that OPG concentrations were higher in RA patients with cardiovascular disease compared to those without. This was true regardless of demographic features, traditional cardiovascular risk factors, adiposity and/or disease characteristics (19).

Regarding vitamin D, observational studies have shown an association between low vitamin D status and risk of cardiovascular events (20). In this context, in a meta-analysis of 19 prospective studies involving the general population (65,994 patients), there was an inverse relationship between serum 25[OH] vitamin D levels and the risk of cardiovascular disease (relative risk of 1.03, 95% CI 1.00-1.60, per 10 ng/mL decrement in serum 25[OH] vitamin D) (21). OPG and vitamin D were, respectively, up- and down-regulated in RA patients vis-à-vis controls (22, 23).

However, the relationship between endothelial function and OPG, vitamin D, and BMD in patients with RA has not been completely elucidated.

Osteoporosis, vascular calcification and cardiovascular events appear to be closely linked, regardless of age. However, the relationship between atherosclerosis and osteoporosis has not been fully studied in RA. In the present study, we assessed BMD by dual x-ray absorptiometry, as well as vitamin D and OPG serum levels, and the presence of subclinical atherosclerosis by flow-mediated vasodilatation of brachial artery in RA patients and matched controls with no previous history of cardiovascular events. We sought to determine whether BMD has some association with endothelial dysfunction in patients with RA, taking into account bone-related metabolites like vitamin D and OPG.

Materials and methods

Study participants

Two hundred and sixteen subjects (111 RA patients and 105 age-matched controls) were recruited for this cross-sectional study. All RA patients were 18 years old or older and fulfilled the 2010 ACR/EULAR diagnostic criteria (24). They had been diagnosed with RA by rheumatologists and were periodically followed at a rheumatology outpatient clinic. In addition, for the purpose of inclusion in the present study, RA disease duration had to be ≥1 year. Because anti-TNF-alpha treatment has been associated with improved endothelial function (25), RA patients undergoing TNF-alpha antagonist therapy were not included in the present study. The control group consisted of patients attending outpatient clinics for osteoarthritis, and who lived in the same area during the same time period of the study. In addition, they were matched for age and comorbidity. The study was performed from December 2011 to November 2012; therefore all seasons were equally spanned in the study interval. Patients and controls who were under vitamin D supplementations, had a history of a previous bone fracture, myocardial infarction, angina, stroke, a glomerular filtration rate <60 ml/min/1.73 m^2, a history of cancer, or any other chronic disease, or evidence of active infection were excluded. None of the controls was receiving glucocorticoids; however, since they are often used in the management of RA, patients taking less than 10 mg/prednisone (or an equivalent dose) were not excluded.

The study protocol was approved by the Institutional Review Committee at Hospital Universitario de Canarias (Spain), and all subjects provided written informed consent.

Data collection

Surveys in RA patients and controls were performed in the same manner, except for the specific management for RA. All subjects completed a cardiovascular risk factor and medication use questionnaire and underwent a physical examination to determine their anthropometrics and blood pressure. Medical records were reviewed to ascertain specific diagnoses and medications. Experienced research nurses obtained all anthropometric measures (weight, standing height, and hip and waist circumferences). In patients with RA, disease activity was measured using the Disease Activity Score (DAS28) in 28 joints (26), while disease disability was determined using the Health Assessment Questionnaire (HAQ) (27).

Vitamin D and osteoprotegerin assessments

Vitamin D was determined through radioimmunoassay (DIAsource ImmunoAssays). Precision was estimated 7.2 to 7.3% (inter-assay) and 7.2 to 8.7% (intra-assay). Vitamin D levels where categorised according to the Institute of Medicine (IOM) systematic review (28) values: deficit (<15 ng/mL), deficiency (15–30 ng/mL), and normal (>30 ng/mL).

Human OPG serum levels were determined by ELISA as previously reported (19). Briefly, 96-well microplates were precoated with anti-human OPG antibody (Peprotech). Recombinant human OPG (Peprotech) was used to prepare the standard curve. The standard dilution series ranged from 0.313 to 20 ng/ml. First, 50 µl of each standard or sample was added to the appropriate wells and incubated for 3 hours at room temperature. After discarding the solution and washing 4 times, 50 µl of prepared biotinylated anti-human OPG antibody (Peprotech) was added to each well and incubated for 1 hour. After washing away unbound biotinylated antibody, 50 µl of horseradish peroxidase (HRP)-conjugated avidin (eBioscience) was pipetted into the wells and incubated for 30 minutes. Finally, plates were developed with ABTS Liquid Substrate (Peprotech) and read at 405 and 600 nm (as reference wavelengths) (19). Standard techniques were used to measure plasma C-reactive protein (CRP), the Westergren erythrocyte sedimentation rate (ESR), and serum lipid levels.

Bone mineral density assessment

Dual x-ray absorptiometric (DXA) measurements of areal BMD (g/cm^2) at the lumbar spine L1–L4 and the femoral neck were carried out using a Lunar DPX Prodigy densitometer (Lunar, Madison, WI, USA). Other regions of interest such as the Ward's triangle, femoral diaphysis, total hip, and trochanter areas were also measured. The short-term precision of DXA measurements was 0.8% for the lumbar spine and 1.5% for the femoral neck. Results for areal BMD were expressed as an absolute value (g/cm^2, bone mineral content relative to projected area) and transformed to a T score using normal standard values from the Spanish population. Arms, legs, trunk, and total body BMD were also assessed and data was expressed as a percentage of body mass (bone mass divided for total body mass) in each of these areas. Cut-off values to categorise individuals were based on the World Health Organization criteria for diagnosing osteoporosis (29) according to the following measures – Normal: a BMD not inferior than -1 SD below young normal (T score ≥1); osteopenia: a BMD between 1 and 2.5 SD below young normal (T score <-1 and >-2.5); osteoporosis: a BMD 2.5 or more SD below young normal (T score ≤-2.5). For the purposes of this study, osteopenia and osteoporosis (T score below <-1 SD) were included in the same category. For patients younger than 50 years old, we used the follow-

ing two categories: 'Within the expected range for age' (Z-score > -2 SD) and 'Below the expected range for age' (Z-score <-2 SD).

Assessment of flow-mediated endothelial-dependent vasodilation of the brachial artery

Flow-mediated endothelium-dependent vasodilatation (FMD) of the brachial artery was assessed by means of ultrasound imaging using a 7-MHz linear probe and automated vessel-diameter measurements (Medical Imaging Applications), as previously described (30). The studies were performed by a single examiner who acquired the images of the brachial artery in the morning, while patients were fasting, in a temperature-controlled room after 10 minutes of rest. Patients fasted for 8 h before the study and all vasoactive medications were withheld for at least four half-lives. In addition, subjects did not exercise or ingest substances that might affect FMD. The brachial artery was imaged above the antecubital fossa continuously for 1 minute at baseline and again after inflation (pressure, 250 mm Hg for 5 minutes) and deflation of a sphygmomanometer cuff placed on the forearm. Images were subsequently analysed offline with the use of dedicated edge detection software (Brachial Tools, Version 3.2.6, Medical Imaging Applications, Coralville, Iowa). Dilatation was quantified as the change, expressed as a percentage, from baseline to the peak diameter, between 45 and 75 seconds after release of the blood-pressure cuff. After 10 minutes of rest, endothelium-independent dilatation was measured after sublingual administration of 25 μg of nitroglycerin according to the same recording protocol.

Statistical analysis

Comparisons between RA patients and controls were performed using a χ^2 test for categorical variables or a Student's *t*-test for continuous variables (data expressed as mean ± standard deviation-SD). For non-continuous variables, either a Mann-Whitney U-test was performed or a logarithmic transformation was made, and data were expressed as a median (interquartile range-IQR).

Table I. Demographic, analytical and disease-related data.

	Control patients (n=105)	RA patients (n=111)	*p*-value
Female, n (%)	97 (92)	100 (90)	0.55
Age, years	55.8 ± 9.5	54.8 ± 10.3	0.44
Anthropometric data			
Height, cm	160 ± 8	160 ± 7	0.72
Weight, kg	73 ± 15	75 ± 15	0.31
Body mass index, kg/m^2	28.3 ± 4.8	29.2 ± 5.7	0.25
Waist circumference, cm	95 ± 12	98 ± 15	0.09
Waist/hip ratio	88 (83-92)	90 (84-96)	0.08
Hip circumference, cm	105 ± 12	108 ± 12	0.58
Bicipital circumference, cm	30 ± 5	30 ± 3	0.88
Comorbidity			
Hypertension, n (%)	37 (35)	44 (40)	0.44
Systolic pressure, mmHg	120 (111-140)	128 (115-140)	0.63
Diastolic pressure, mmHg	80 (70-82)	80 (70-80)	0.55
Current smoker, n (%)	5 (5)	5 (4)	0.74
Diabetes, n (%)	8 (8)	16 (14)	0.11
Menopause, n (%)	69 (66)	71 (64)	0.98
Currently on aspirin, n (%)	13 (12)	7 (6)	0.13
NSAID use, n (%)	1 (10)	16 (14)	0.27
Other chronic illness, n (%)	29 (28)	18 (16)	0.05
Analytical data			
ESR, mm/h	23 ± 14	29 ± 20	**0.02**
CRP, mg/L	3.75 ± 3.06	8.27 ± 16.30	**0.01**
Cholesterol, mg/dL	205 ± 36	210 ± 41	0.49
Triglycerides, mg/dL	112 ± 57	132 ± 76	0.18
HDL cholesterol, mg/dL	55 ± 11	56 ± 15	0.74
LDL cholesterol, mg/dL	128 ± 32	129 ± 35	0.91
Apoliprotein A1, mg/dL	150 ± 19	151 (141-165)	0.44
Apoliprotein B, mg/dL	87 ± 25	90 ± 17	0.65
Brachial echography			
FMD%	8.5 (4.5-15.6)	5.3 (0.0-9.2)	**0.00**
Rheumatoid arthritis-related data			
DAS28		3.54 ± 1.04	
HAQ		0.750 (0.250-1.500)	
Current prednisone intake, n (%)		49 (44)	
Prednisone, mg/day		2 (0-5)	
Disease duration, years		8 (4-13)	
Rheumatoid factor, n (%)		60 (54)	
Methotrexate, n (%)		89 (80)	

Values are expressed as the mean ± standard deviation or median (interquartile range).
ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; HAQ: Health Assessment Questionnaire; DAS28: Disease Activity Score; HDL: high-density lipoprotein; LDL: low-density lipoprotein. FMD%: flow-mediated dilation; NSAID: non-steroidal anti-inflammatory drugs.

The relationship between BMD and endothelial function was carried out using multivariate analysis, adjusting for factors known to be associated with cardiovascular disease and for bone metabolism proteins. For this purpose, BMD was categorised as a dummy (binary) variable and considered superior or inferior to -1 T-score SD. The independent variable was categorised in this way since it does not have a lineal distribution and in our study few patients fulfilled the criteria to be included within the osteoporosis category. We established several models: Model 1, which studied the relationship between BMD and FMD adjusted for age, sex and body mass index; Model 2 was adjusted for the variables of Model 1 plus hypertension, diabetes, lipids, and smoking; and Model 3, which was adjusted for the variables of Models 1 and 2 plus serum levels of OPG and vitamin D. Figure 1 represents the relationship of OPG and vitamin D with FMD after adjusting for age, sex, body mass index, hypertension, diabetes, smoking and, in this case, BMD. Tukey box plots, which represented the interquartile range and the 75th and 25th percentiles plus 1.5 times the interquartile range (whiskers) were included in Figure 1 to show

Table II. Bone mineral density and serum osteoprotegerin and vitamin D differences between controls and rheumatoid arthritis patients.

	Controls	RA patients	*p*-value
WHO classification, n (%)			
Patients >50 years old			
Normal (T-score >-1 SD)	49 (46.4)	38 (34.4)	0.08
Osteopenia (T-score -1– -2.5 SD)	43 (41.1)	64 (57.4)	**0.05**
Osteoporosis (T-score <-2.5)	13 (12.5)	9 (8.2)	0.70
Patients <50 years old			
Within the expected range for age	105 (100)	106 (95.5)	0.43
Below the expected range for age	0 (0)	5 (4.5)	
T-scores, n (%)			
L1L4 T-score			
>-1 SD	70 (66.7)	71 (63.9)	
<-1 SD	35 (33.3)	40 (36.0)	0.67
Femoral neck T-score			
>-1 SD	77 (73.3)	69 (62.1)	
<-1 SD	28 (26.7)	42 (37.8)	0.14
Ward's triangle T-score			
>-1 SD	49 (46.7)	54 (48.6)	
<-1 SD	56 (53.3)	57 (51.4)	0.81
Trochanter T-score			
>-1 SD	90 (85.7)	82 (73.9)	
<-1 SD	15 (14.3)	29 (26.1)	**0.05**
Total hip T-score			
>-1 SD	85 (80.9)	80 (72.1)	
<-1 SD	20 (19.0)	31 (27.9)	0.20
*Grams/cm² (± SD)**			
L1-L4	1.111 ± 0.208	1.089 ± 0.166	**0.02**
Femoral neck	0.901 ± 0.150	0.884 ± 0.132	0.11
Ward's triangle	0.730 ± 0.169	0.710 ± 0.141	0.13
Femoral trochanter	0.806 ± 0.143	0.782 ± 0.143	**0.03**
Femoral diaphysis	1.199 ± 0.220	1.164 ± 0.180	**0.04**
Total hip	0.994 ± 0.164	0.965 ± 0.147	**0.02**
*Bone mass (± SD)**			
Arms bone mass (%)	4.1 ± 0.8	3.8 ± 1.0	**0.04**
Legs bone mass (%)	12.8 ± 2.0	12.1 ± 2.0	**0.02**
Trunk bone mass (%)	11.1 ± 2.5	9.9 ± 2.4	**0.00**
Total bone mass percentage (%)	34.7 ± 5.7	32.2 ± 5.8	**0.00**
Analytical data			
Osteoprotegerin, ng/mL	0.84 (0.50-1.46)	1.52 (0.96-3.40)	**0.00**
Vitamin D, ng/mL	42.50 (31.27-48.13)	33.64 (27.27-48.28)	0.10
Normal (>30 ng/mL), n (%)	82 (78.1)	72 (62.6)	**0.03**
Deficiency (15-30 ng/mL), n (%)	19 (18.1)	40 (34.8)	**0.02**
Deficit (<15 ng/mL), n (%)	4 (3.8)	3 (2.6)	0.99

*Grams/cm² and bone mass analysis were adjusted for age, sex and body mass index. Values are expressed as the mean ± standard deviation or median (interquartile range). SD: standard deviation; BMD: bone mineral density; L1-L4: Lumbar vertebrae 1 to 4. Below the expected range for age: Z-score <-2 SD. Within the expected range for age: (Z-score >-2 SD).

the relationship of vitamin D and OPG, as expressed in quartiles with FMD in both patients and controls. The *p*-values expressed the linear trends of FMD following OPG and vitamin D stratification (quartiles) using linear regression with orthogonal polynomial codification adjusted for sex, age, body mass index, diabetes, hypertension, lipids, smoking, and percentage of body bone mass. All analyses used a 5% two-sided significance level and were performed using SPSS software, version 21 (IBM, Chicago, IL, USA). A *p*-value <0.05 was considered statistically significant.

Results

Demographic, analytical and disease-related data

Two hundred and sixteen subjects (111 RA patients and 105 controls) with a mean (±SD) age of 54.8±10.3 years and 55.8±9.5 years, respectively (*p*=0.44), were included in this study.

Demographic and disease-related characteristics of the participants are shown in Table I. There were no differences in the frequency of hypertension and diabetes between patients and controls. RA patients had moderately active disease as shown by DAS28 (3.54±1.04) and displayed a median HAQ of 0.750 (IQR 0.250-1.500). Almost half (44%) were taking prednisone (median current dose 2 [0–5] mg/day). As expected, the ESR and CRP levels were significantly higher in patients compared to controls. The lipid profile did not display significant differences between patients and controls. Nevertheless, FMD values were higher in controls than in RA patients (8.5 [4.5–15.6] % *vs*. 5.3 [0.0–9.2] %, *p*=0.00).

BMD, and vitamin D and OPG plasma levels in patients and controls

Differences in BMD, OPG and vitamin D serum levels between patients and controls are shown in Table II. Controls more commonly had normal BMD values according to the WHO classification system (29) than RA patients (46.4% in controls *vs*. 34.4% in patients), although the difference fell slightly out of the range of significance (*p*=0.08). Although the frequency of osteoporosis in RA patients did not differ from that of controls (12.5% *vs*. 8.2%, *p*=0.70), the frequency of osteopenia was marginally higher in patients compared to controls (57.4% *vs*. 41.1%, *p*=0.048).

The categorisation of subjects younger than 50-years-old, using the definitions of "within the expected range for age" (Z-score >-2 SD) and "below the expected range for age" (Z-score <-2 SD), did not disclose statistically significant differences. When the adjusted analysis was made using T-score data (T-score higher or lower than -1 SD) or grams/cm² in specific areas, RA patients showed lower values, although statistically significant differences were not attained in all areas. In contrast, when differences in the percentage of body bone mass composition were assessed, RA patients showed statistically significantly lower percentages of bone mass than controls (Table II). In this regard, all the body areas including the arms, legs, trunk, and total body bone mass percentage (32.2±5.8 *vs*. 34.7±5.7%, *p*=0.00) were lower in RA patients than in controls.

Table III. Relationship of BMD with FMD in RA patients and controls.

	% FMD, beta coefficient (95% CI)			
	Model 1			
	Controls	*p*-value	RA patients	*p*-value
L1L4 T-score < -1 SD	-3.86 (-12.63-4.91)	0.37	1.46 (-6.58-9.50)	0.72
Femoral neck T-score < -1 SD	-8.87 (-17.96-0.23)	**0.06**	1.33 (-6.80-9.45)	0.75
Ward's triangle T-score < -1 SD	-5.02 (-13.12-3.09)	0.21	-2.41 (-10.14-5.33)	0.54
Throchanther T-score < -1SD	-9.96 (-19.35-0.57)	**0.04**	-2.59 (-11.65-6.47)	0.57
Total hip T-score < -1 SD	-8.87 (-17.96-0.23)	**0.06**	-2.58 (-12.48-7.32)	0.60
	Model 2			
	Controls	*p*-value	RA patients	*p*-value
L1L4 T-score < -1 SD	-4.79 (-14.93-5.36)	0.34	1.58 (-7.14-10.31)	0.72
Femoral neck T-score < -1 SD	-10.20 (-20-41-0.01)	**0.05**	1.47 (-7.05-9.99)	0.73
Ward's triangle T-score < -1 SD	-5.59 (-14.91-3.72)	0.22	-2.89 (-11.07-5.29)	0.48
Throchanther T-score < -1SD	-10.96 (-21.37-0.55)	**0.04**	-3.26 (-13.05-6.54)	0.51
Total hip T-score < -1 SD	-10.20 (-20.41-0.01)	**0.05**	-3.20 (-13.55-7.15)	0.54
	Model 3			
	Controls	*p*-value	RA patients	*p*-value
L1L4 T-score < -1 SD	.2.47 (-11.76-6.82)	0.58	1.16 (-9.37-11.69)	0.82
Femoral neck T-score < -1 SD	-8.73 (-16.88-0.34)	**0.05**	1.25 (-9.98-12.47)	0.82
Ward's triangle T-score < -1 SD	-3.61 (-12.24-5.02)	0.38	-3.50 (-15.25-8.24)	0.55
Throchanther T-score < -1SD	-9.67 (-19.37-0.03)	**0.04**	-6.28 (-19.75-7.20)	0.35
Total hip T-score < -1 SD	-8.27 (-16.88-0.34)	**0.05**	-4.61 (-17.71-8.48)	0.48

T-score in each bone area has been categorised in < -1 SD and > -1 SD. Beta coefficient represents the change in FMD% from > -1 SD to < -1 SD. Model 1; data adjusted for age, sex and body mass index. Model 2; data adjusted for model 1 + hypertension, diabetes and smoking. Model 3; data adjusted for model 1 + model 2 + vitamin D and osteoprotegerin (p<0.10) are depicted in bold.

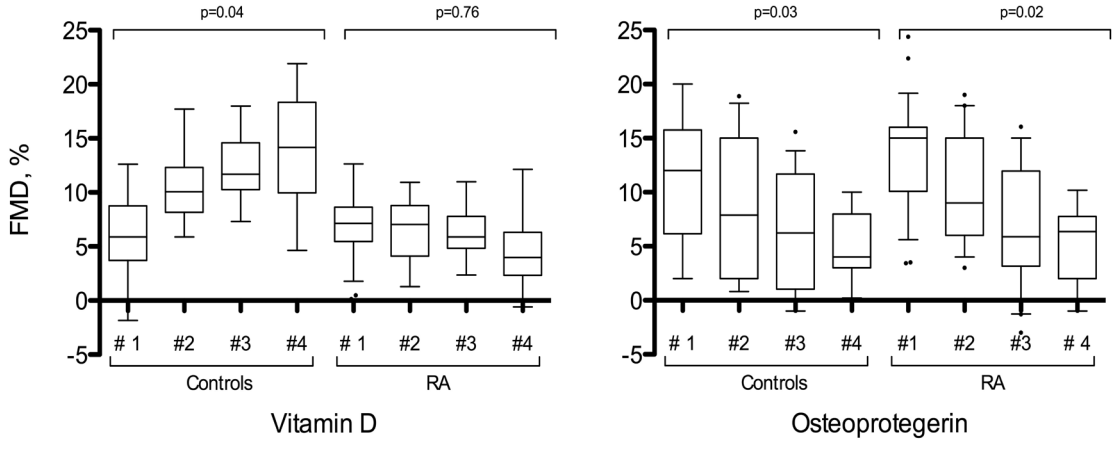

Fig. 1. Relationship of OPG and vitamin D with FMD after adjusting for age, sex, body mass index, hypertension, diabetes, smoking and BMD.

OPG serum levels were higher in RA patients compared to healthy controls (1.52 [IQR 0.96–3.40] *vs*. 0.84 [0.50–1.46] ng/mL, p=0.00). RA patients tended to have lower levels of vitamin D (33.64 [IQR 27.27–48.28] *vs*. 42.50 [31.27–48.13] ng/mL, p=0.10). However, when subjects were stratified according to vitamin D levels, we observed that vitamin D deficiencies were more common in RA patients than in controls (34.8 *vs*. 18.1 %, p=0.02) (Table II).

Association of BMD, vitamin D and OPG serum levels with endothelial dysfunction

Table III shows the relationship of BMD with FMD. BMD inferior to -1 T-score SD was associated with lower values of FMD in controls, though not in RA patients. Moreover, in controls femoral neck, throchanter and total hip BMD scores were associated with significantly lower FMD values. This was not the case for other areas such as the lumbar spine or Ward's triangle. When this analysis was performed in RA patients, neither hip, lumbar nor Ward's areas were associated with FMD. These results were replicated when adjusting for cardiovascular risk factors (diabetes, hypertension, serum lipids, and smoking) and for the bone mineral-related proteins OPG and vitamin D. After these corrections, the association of BMD with FMD remained statistically significant in controls. However, in RA patients the absence of any association between BMD and FMD per-

sisted even after making adjustments that included RA-related data such as steroid intake, ESR, C-reactive protein or disease activity or duration (data not shown in Table III).

Figure 1 shows a trend analysis (adjusted for classical cardiovascular risk factors, body mass index and bone mass percentage) examining the relationship of OPG and vitamin D as expressed in quartiles with FMD. In both RA patients and controls low FMD was associated with high levels of OPG. In contrast, high vitamin D levels were associated with high FMD values in controls but not in RA patients.

Discussion

Endothelial dysfunction is typically present in long-standing RA patients, even in those without traditional cardiovascular risk factors (31, 32). The present study supports the relationship between OPG and endothelial function in RA. However, our results do not shed light on the relationship between vascular damage and osteoporosis in RA because endothelial function, as measured by FMD, showed no association with vitamin D levels and BMD in RA patients. This finding was somehow unexpected due to the fact that in a multivariate analysis endothelial function was found to be related to BMD and vitamin D levels in controls.

Low levels of vitamin D are common in patients with RA. Several studies have shown a high prevalence of suboptimal vitamin D levels in these individuals (22, 33). Moreover, 25(OH) not only have vitamin D plasma levels been inversely correlated with RA disease activity (34), but they have also been associated with cardiovascular disease in the general population (35, 36). As pointed out by Dessein (37), vitamin D may be involved in both high-grade systemic inflammation and in the enhanced cardiovascular risk factors affecting insulin resistance, endothelial activation, and lipids. However, in our series endothelial function, as measured by FMD, was not associated with vitamin D levels or BMD. This finding was equally unexpected since in a multivariate analysis endothelial function was found to be related to BMD and vitamin D levels in controls. The reasons for this disparate association in patients with RA and controls remain unknown. Haque *et al*. (38) suggested that vitamin D deficiency may be independently associated with several cardiometabolic intermediates in RA patients including HDL-cholesterol, HOMA-IR index (homeostatic model assessment), fibrinogen, E-selectin, and soluble intercellular adhesion molecule. Another study (39) also revealed an association between serum vitamin D levels and traditional cardiovascular risk factors such as serum low-density lipoprotein cholesterol, triglycerides, and the metabolic syndrome observed in patients with RA. However, these two studies lacked a control group and endothelial function was not assessed. Interestingly, in another study (40) involving 87 vitamin D-deficient RA patients (but no control group), vitamin D levels in the former (<20 ng/ml, n=25) showed a correlation with microvascular function when assessed using the reactive hyperemia index. Nevertheless, this association was not found in RA patients with insufficient (<30 ng/ml) or normal vitamin D levels. An explanation for our findings might be that the role played by vitamin D in cardiovascular risk may be less relevant in RA patients than in the general population. Thus, it is possible that the presence of a chronic proinflammatory condition might prevail over other potential factors that promote the development of vascular damage in RA.

Recent clinical studies in the general population have demonstrated that increased concentrations of OPG are associated with the presence and severity of coronary artery disease (15, 41, 42). It has also been found that OPG is upregulated in RA patients and appears to be associated with the inflammation already present in the existing disease (23, 43). Unselected patients with RA exhibit higher serum levels of OPG than do controls(19) and TNF-α blockade can reduce OPG concentrations in RA (18, 44). Our results in patients with RA are in keeping with previous reports that showed OPG to be independently associated with carotid plaque and carotid intima-media thickness (18, 45) and coronary-artery calcification (23) in RA patients. Due to the cross-sectional nature of our study, however, we cannot completely exclude the possibility that increased levels of OPG in RA patients could stem from a compensatory effect meant to counteract the mechanisms of accelerated atherogenesis that occur in this chronic proinflammatory disease. In any case, this assumption seems to be unlikely as this relationship between OPG and endothelial function was similarly observed in controls who exhibited a lower inflammatory burden than their RA patient counterparts. What is novel in our study is that the relationship between OPG and FMD in RA patients was independent of BMD, and therefore is not mediated by the low BMD that RA patients often exhibit. In this regard, our results addressing BMD in RA were in keeping with previous reports that recorded significantly lower BMD levels in patients with RA (46, 47).

In keeping with previous studies (8, 10, 48, 49), we also found endothelial function to be independently associated with BMD in non-RA individuals. Remarkably, we observed that this association between BMD and FMD in controls occurred independently of OPG and vitamin D levels. As we did not find such a relationship in RA patients, we can only state that the lack of any association between BMD and FMD in RA patients was not due to such confounding factors as OPG or vitamin D plasma levels.

In a study that included 47 patients with RA, Tanaka *et al*. demonstrated that trabecular BMD at the distal radius was a significant factor independently associated with greater femoral-ankle and brachial-ankle pulse wave velocity when adjusted for age, blood pressure, and smoking (50). Interestingly, as with our own findings, these authors detected no such association when total bone mass (cortical plus trabecular) was analysed. However, unlike the study by Tanaka *et al*., we assessed FMD in a larger series of RA patients, one which was not restricted to postmenopausal women. Moreover, we measured total body bone mass instead of distal radius and adjusted for two relevant bone metabolites, OPG and vitamin D.

We acknowledge that some possible limitations may exist in our study. In this regard, age was not an exclusion criterion in our study as we enrolled pre- and menopausal women. In addition, some potential confounding factors such as physical activity, diet calcium intake and sun exposure were not assessed in the present study.

Taken together, the findings in our study indicate that OPG serum level is an independent factor associated with endothelial function in RA patients and controls, whilst vitamin D levels and BMD showed such a relationship only in healthy controls. Remarkably, the association of OPG with FMD occurred independently of BMD status.

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