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Original Article

Association Between Homocysteine Serum Level and Bone Mineral Density in Patients With Rheumatoid Arthritis

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease with unknown etiology that primarily affects the peripheral joints and, over time, leads to loss of mobility if untreated *(1)*. RA is an important risk factor of osteoporosis and occurrence of fractures *(2,3)*. Many authors describe a significantly higher frequency of osteoporosis and fractures in patients with RA in comparison to a control group *(4)*. The etiology of progressive bone mass loss in RA patients is multifactorial and remains obscure. Factors related to the pathological process – activity of RA and applied therapy – seem to be significant *(5)*.

Homocysteine (Hcy) is a sulfhydryl containing amino acid produced by demethylation of an essential amino acid (methionine). Methylation of Hcy, catalyzed by methionine synthetase produces methionine. Folate and vitamin B12 are essential components in the metabolism of Hcy, which occurs through remethylation to methionine or trans-sulfuration to cysteine. The enzyme methylene-tetrahydrofolate-reductase (MTHFR) is responsible for the reduction of 5,10-methylene-THF to 5-methyl-THF, where vitamin B12 acts as a cofactor *(6)*.

Results from recent investigations show an association between increased plasma concentrations of Hcy and reduced bone mineral density (BMD) and increased risk of bone fractures *(7–12)*. Actually, hyperhomocysteinemia (HHcy) has been considered as a new independent risk factor for osteoporosis and fractures, especially in the elderly *(13)*.

Several mechanisms have been proposed to link HHcy with the pathogenesis of osteoporosis: reduction of bone formation by inducing apoptosis in human bone marrow stromal cells *(14)*, osteoclastogenesis stimulation *(15)*, bone blood flow reduction and consequently changes on bone biomechanical properties *(16)*, decline in physical functioning, alteration of gene expression with reduced methylation capacity *(17)*, oxidative damage on trabecular structures *(18)*, and reduction of bone strength by interfering with collagen crosslinks *(10,19)*.

In the present study we compared plasma Hcy levels of patients with RA with those of healthy controls. We also assessed if an association between Hcy and BMD exists in patients with RA.

Materials and Methods

Patient Selection

The study was performed in Vega-Baja Hospital, Orihuela (Spain) from November 2016 to May 2018. We prospectively enrolled 63 consecutive women patients affected by RA and 60 matched healthy women controls. All patients included in this study had normal serum creatinine (Cr) levels and met the 2010 American College of Rheumatology/European League Against Rheumatism criteria for RA *(20)*.

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At the clinic visit, participants completed questionnaires about their medical history, current medication used, and lifestyle characteristics. Informed consent was obtained for all subjects, and the study was approved by the local ethics committees and conducted in accordance with the guidelines in the Declaration of Helsinki.

Weight and height were measured with a balanced scale and attached stadiometer (Seca model 700-220, Vogel & Halke Gmbh & Co., Hamburg, Germany) in light indoor clothing without shoes before performing dual-energy X-ray absorptiometry (DXA), and body mass index (BMI) was calculated as kilograms per square meters (kg/m^2).

Disease severity was scored through disease activity score of 28 joints. Current smokers were defined as those who reported having smoked ≥1 cigarette per day regularly during the year preceding the examination. Kidney function was assessed using the estimated glomerular filtration rate (eGFR) calculated by the CKD-Epi study equation *(21)*.

Information regarding previous fractures was obtained through patient self-reporting questionnaires during routine clinic visits and spinal X-ray evaluations.

BMD Measurement

Total hip, femoral neck, and lumbar spine (L1−L4) BMD was measured in all subjects included in the study by DXA using the same Hologic DXA scanner (model QDR 4500) and were expressed in absolute values as grams of mineral content per square centimeters of bone area (g/cm^2), T-scores (standard deviation [SD] compared to a Caucasian female normative database), and Z-score (SD compared to the subject's age and gender). Quality control of the scanner was performed daily by measuring a phantom's density that showed stable results at the time of the study. Coefficients of variation of DXA measurements were 1%. Consistent with World Health Organization classification system, osteoporosis is defined as T-score at least −2.5 SDs below the mean (≤−2.5 SD) and osteopenia or low bone mass is defined as a T-score between −2.5 and −1.0 SDs below the mean.

Osteoporotic Fracture Risk Evaluation by FRAX

The FRAX scoring tool was applied using the on-line calculator (www.shef.ac.uk/FRAX); clinical and BMD data were used.

Laboratory Measurements

In all the cases, a blood sample was taken in the morning after an overnight fasting and stored at −70°C until the assays.

The sera were tested for creatinine, calcium, phosphorus, intact parathyroid hormone (iPTH), 25-hydroxy vitamin D (25-(OH)D), C-reactive protein (CRP), anticitrullinated protein antibodies (ACPAs), rheumatoid factor (RF), and Hcy. Creatinine was determined by Jaffe method (Siemens Healthcare Diagnostic Inc., NY). Calcium and phosphorus were determined colorimetrically using commercial reagents in an automated chemical analyzer (Siemmens Healthcare Diagnostic Inc., NY). The iPTH and 25-(OH)D were measured by chemiluminescent assays (Dia Sorin. Saluggia, Italy). CRP was measured by turbidimetric immunoassay (Siemmens Healthcare Diagnostic Inc., NY). ACPAs were detected using a second-generation ELISA (ACPAs) kit (ORGENTEC Diagnostika GmbH. Mainz, Germany) while IgM RF was determined as part of routine analysis by turbidimetric assay (Siemmens Healthcare Diagnostic Inc., NY) according to the manufacturers' instructions. Serum Hcy (Siemmens Healthcare Diagnostic Inc., NY) were measured by immunonephelometric method according to the manufacturer's recommendations. All other routine serum biochemistries were measured at the Department of Clinical Chemistry, Vega-Baja Hospital.

Statistical Analysis

Data were analyzed by statistical software SPSS 18 (Chicago, IL) and with the program R version Rx 64.3.5.0 (Vienna, Austria), using independent samples *t* test, Mann-Whitney *U* test, and chi-square test where appropriate. Spearman's coefficient and Pearson's correlation were calculated as suitable to determine the correlation between the bio-chemical parameters. Multiple regression analysis was performed to determine the relationship between BMD at different regions, age, smoking, BMI, weight, as well as serum levels of Hcy, iPTH, and 25-(OH)D. *p* Values of less than 0.05 were considered statistically significant. The quantitative data were shown as mean ± SD and median (Q1−Q3) as suitable.

Results

Characteristics of the Study Subjects

The characteristics of the study subjects are shown in Table 1. A total of 63 female patients were included in our study, with a mean (SD) age of 53 ± 8 yr. The majority were Caucasian (90.5%). Thirty-four patients were menopausal and 29 nonmenopausal patients. The mean duration of RA was 8.5 ± 5.8 yr. The mean disease activity scores in 28 joints according to the erythrocyte sedimentation rate (ESR) indicated low disease activity 3.0 ± 1.3. The mean health assessment questionnaire was 0.75 ± 0.67. Twenty-eight patients were treated with methotrexate, with a median weekly dose of 11.5 ± 4.8 mg, all the patients received 10 mg folic acid supplementation per week. Sixteen patients were treated with prednisone with a median daily dose of 6.5 ± 3.5 mg. Thirty-two patients were treated with biological agent (9 with etanercept, 9 with certolizumab pegol, 7 with tocilizumab, 6 with adalimumab, and 1 with rituximab). According to the lowest T-score in any of the skeletal sites where BMD was measured, 11 subjects had osteoporosis, 15 had low bone mass, and 37 had normal BMD values. Eight patients (12.7%) had fractures, 6 of which were vertebral.

Table 1
Characteristics of Patients With Rheumatoid Arthritis and Healthy Control

	RA	HC	
	Mean ± SD	Mean ± SD	*p* value
Age, yr	53.1 ± 8.3	52.7 ± 9.7	0.80
Height, cm	160.8 ± 6.2	169.9 ± 7.1	<0.001
Body weight, kg	68.7 ± 14.5	67.2 ± 12	0.52
Body mass index, kg/m^2	26.5 ± 5.6	25.9 ± 4.3	0.49
Duration of RA, yr	8.5 ± 5.8	-	-
DAS28-ESR	3.0 ± 1.3	-	-
HAQ	0.75 ± 0.67	-	-
Smoking, *n* (%)	14 (22.2)	18 (30)	0.47
Diabetes mellitus, *n* (%)	3 (4.7)	2 (3.3)	0.62
Prednisone, mg/d	6.5 ± 3.5	-	-
Methotrexate, mg/wk	11.5 ± 4.8	-	-
Biological agent use, *n* (%)	32 (50.7)	-	-
Fragility fractures, *n* (%)	8 (12.7)	2 (3.3)	0.04
FRAX-BMD, MOF	5.3 (3.6)	4.9 (2.6)	0.47
FRAX-BMD, HF	0.9 (1.4)	0.6 (1)	0.16
BMD (g/cm^2)			
Lumbar spine	1.01 ± 0.18	0.94 ± 0.18	0.02
Femoral neck	0.79 ± 0.11	0.87 ± 0.15	<0.001
Total femur	1.13 ± 0.63	1.06 ± 0.22	0.40
T-score			
Lumbar spine	-0.46 ± 0.78	-0.9 ± 1.3	0.01
Femoral neck	-0.49 ± 1	-0.7 ± 1.4	0.32
Total femur	-0.28 ± 0.89	-0.4 ± 1.2	0.51
Z-score			
Lumbar spine	0.57 ± 1	0.1 ± 1.2	0.01
Femoral neck	0.40 ± 0.98	0.2 ± 1.2	0.30
Total femur	0.46 ± 0.47	0.3 ± 1.1	0.33

Abbr: BMD, bone mineral density; DAS, disease activity score; HAQ, Health Assessment Questionnaire; FRAX-BMD, BMD adjusted FRAX; HC, healthy control; HF, hip fracture; MOF, major osteoporotic fracture; RA, rheumatoid arthritis; SD, standard deviation.

A total of 60 healthy women controls were included in our study, with a mean (SD) age of 52 ± 9 yr. The majority were Caucasian (98.3%). Thirty-one were menopausal and 29 nonmenopausal. According to the lowest T-score in any of the skeletal sites where BMD was measured, 9 subjects had osteoporosis, 20 had low bone mass, and 31 had normal BMD values. Two controls (3.3%) had fractures, none of which were vertebral.

Laboratory Results

Serum biochemistries of the patients and healthy controls are presented in Table 2. The mean serum total CRP, ACPAs, and RF in RA patients were 0.64 ± 0.88 mg/dL, 571.22 ± 1040.89 U/mL, 173.73 ± 751.8 U/mL, respectively. Mean serum total creatinine, calcium, phosphate, 25-(OH)D, and iPTH in RA patients were 0.58 ± 0.11 mg/dL, 9.4 ± 0.3 mg/dL, 3.5 ± 0.5 mg/dL, 24.2 ± 11.4 ng/mL, and 58.5 ± 22.9 pg/mL, respectively. Mean eGFR in RA patients was 93 ± 17.2 mL/min.

Table 2
Serum Homocysteine and Serum Biochemistries of the Patients and Healthy Control

	RA	HC	
	Mean ± SD	Mean ± SD	P-value
Homocysteine, μmol/L	9.93 ± 2.79	7.1 ± 1.4	<0.001
Serum phosphate, mg/dL	3.5 ± 0.5	3.4 ± 0.5	0.25
Serum calcium, mg/dL	9.4 ± 0.3	9.4 ± 0.4	1.00
Alkaline phosphatase, IU/L	89.0 ± 25.5	72.9 ± 19.5	<0.001
25-(OH)D, ng/mL	24.2 ± 11.4	23.1 ± 7.3	0.51
Intact PTH, pg/mL	58.5 ± 22.9	54.9 ± 19.6	0.33
Serum creatinine, mg/dL	0.58 ± 0.11	0.7 ± 0.2	<0.001
eGFR, mL/min	93 ± 17.2	97 ± 13.2	0.14
CRP, mg/dL	0.6 ± 0.8	0.2 ± 0.1	<0.001
ESR, mm/h	23.9 ± 15.8	11.3 ± 10.2	<0.001
RF positive, *n* (%)	46 (73)	-	-
ACPAs positive, *n* (%)	45 (71.4)	-	-

Abbr: 25-(OH)D, 25-hydroxy vitamin D; ACPAs, Anticitrullinated protein antibodies; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sedimentation rate; HC, healthy control; PTH, parathyroid hormone; RA, rheumatoid arthritis; RF, rheumatoid factor; SD, standard deviation.

Serum Hcy concentrations were significantly higher in the RA patients than those in the control group: (9.93 [5.6−18.8] vs 7.11 [4.9−11.1], pg/mL; $p < 0.001$).

Osteoporosis and Risk Factors

Patients had a mean BMI of 26.58 ± 5.61 kg/m^2, smoking status (14 patients: 22%), and diabetes mellitus (3 patients: 3.4%).

Sixteen patients (25.4%) had received corticosteroid treatment within the last 6 mo before the inclusion, with a median dose of prednisone of 6.5 ± 3.5 mg/d.

Correlation Between Hcy Levels and Characteristic of the Rheumatoid Arthritis

Table 3 shows the correlation coefficients between Hcy and other markers in RA patients. We did not find

Table 3
Correlations Between Homocysteine and Study Parameters in RA Patients

	Correlation coefficient	*p*
Age	0.16	0.210
Height	−0.01	0.942
Body weight	−0.06	0.663
Body mass index	−0.07	0.580
Smoking	−0.01	0.898
Disease duration of RA	−0.21	0.098
DAS28-ESR	−0.03	0.790
HAQ	0.02	0.871
Methotrexate dose	0.16	0.410
Prednisone dose	0.56	0.227
Biological agent use	−0.09	0.445
RF	0.09	0.480
ACPAs	0.22	0.090
C-reactive protein	−0.07	0.573
Erythrocyte sedimentation rate	−0.05	0.698
Serum creatinine	0.39	0.001
Serum urea	−0.21	0.109
eGFR	−0.42	<0.001
Serum phosphate	0.08	0.556
Serum calcium	−0.14	0.291
25-(OH)D	0.03	0.841
Intact PTH	−0.17	0.193
FRAX-BMD, MOF	0.06	0.600
FRAX-BMD, HF	0.15	0.218
Fragility fractures	−0.08	0.541

Abbr: 25-(OH)D, 25-hydroxy vitamin D; ACPAs, anticitrullinated protein antibodies; BMD, bone mineral density; DAS, disease activity score; eGFR, estimated glomerular filtration rate; FRAX-BMD, BMD adjusted FRAX; HAQ, Health Assessment Questionnaire; HF, hip fracture; MOF, major osteoporotic fracture; PTH, parathyroid hormone; RA, rheumatoid arthritis; RF, rheumatoid factor.

correlation between Hcy and CRP, ESR, ACPAs, RF, disease activity scores in 28 joints-ESR or duration of RA.

Correlation Between Hcy Levels and Osteoporosis Risk Factors

Higher plasma Hcy concentrations were significantly associated with lower BMD of lumbar spine (Fig. 1) and femoral neck (Fig. 2) in RA patients, Table 4 shows the correlation coefficients between Hcy and BMD. We found a correlation between higher plasma Hcy concentrations and lower eGFR. A positive correlation between Hcy serum level and creatinine plasma level was also observed. However, Hcy levels did not show correlation with the use of prednisone, age, smoking, BMI, 25-(OH) D, iPTH, total calcium and phosphate levels, FRAX-BMD, or fragility fractures.

Multiple linear regression analysis in Table 5 shows the possible influence of age, BMI, smoking, weight, serum Hcy, iPTH, 25-(OH)D levels, and prednisone use in predicting BMD.

Discussion

Osteoporosis is a significant complication of RA, having an additional negative effect on patients' quality of life. Progressive bone mass reduction is a result of numerous factors. Some of them are known, and their effect is confirmed. They are old age, gender, postmenopausal period of life, unbalanced diet, or tobacco smoking. In our study, 16 patients were treated with prednisone with a median daily dose of 6.5 ± 3.5 mg. These patients did not have significantly different BMD when compared to those not receiving glucocorticoids. Glucocorticoid-induced osteoporosis is a well-known clinical entity and is also considered a major risk factor for osteoporosis in patients with RA. However, the impact of low-dose glucocorticoid use on bone health, in patients with active RA, is more complex. The anti-inflammatory effects of glucocorticoids may have beneficial effect on RA disease activity and course, which may compensate for their direct negative effect on bone health. Blavnsfeldt et al *(22)* in a systematic review and meta-analysis of randomized, controlled trials, found no difference in change in BMD between patients receiving up to 24 mo of treatment with prednisone/prednisolone vs placebo, suggesting that in a population with early and active RA, at least through 24 mo, the effects of glucocorticoids on dampening systemic inflammation may counterbalance their adverse effects on bone formation and resorption. Studies completed by numerous authors have demonstrated a significantly higher prevalence of osteoporosis in the group of RA patients compared to healthy controls *(4)*. In fact, in our study, 12.7% of patients with RA presented fragility fractures vs only 3.3% of the control group, and this despite the fact that the FRAX-index showed a low risk of fracture. However, there are still doubts about the etiopathogenesis of osteoporosis in RA patients.

A high serum level of Hcy has been recently recognized as a risk factor for osteoporosis and osteoporotic fractures in postmenopausal women *(7,12,13)*. The role of Hcy is yet not clearly understood, despite the fact that there are several suggested mechanisms for the effect of HHcy in bones *(10,14–16,18)*. Earlier studies that observed high prevalence of osteoporosis in patients with homocysteinuria *(23)*, followed by studies investigating the influence of MTHFR polymorphism in BMD *(24,25)* were forerunners of more recent studies that investigated

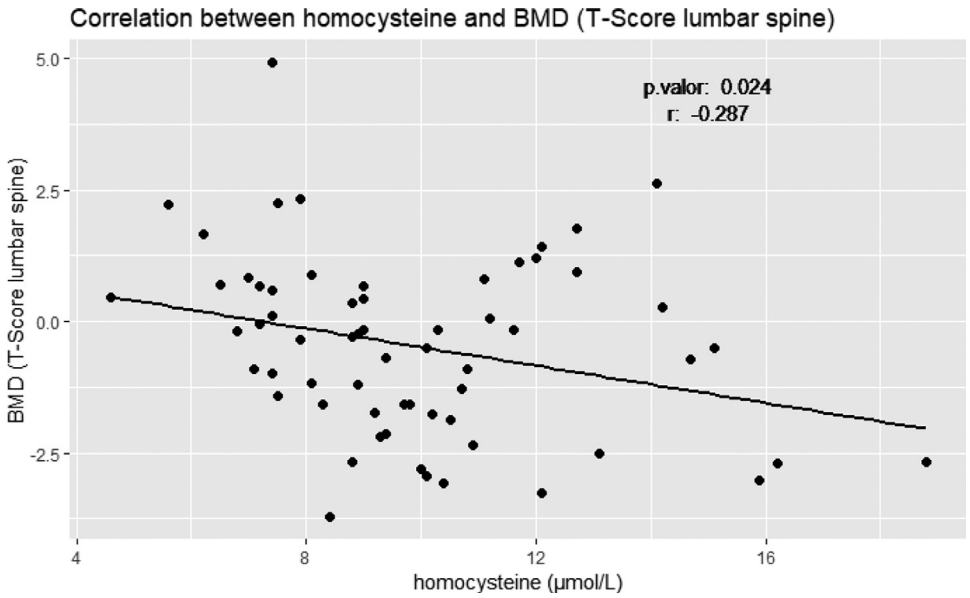

Fig. 1. Correlation between homocysteine and bone mineral density (BMD; T-score lumbar spine).

the relationship between serum levels of Hcy and BMD *(9,11,12,26,27)*. Nevertheless, the inconsistencies persist as to whether HHcy is associated with an increased risk of developing osteoporosis.

In our study, we found significantly elevated plasma Hcy levels in patients with RA compared with healthy controls. Clinical studies reported elevated plasma levels of Hcy in patients with RA *(28,29)*. This HHcy among RA patients seems to be the result of both genetic and nongenetic factors associated with the disturbance of Hcy metabolism *(30)*. Genetic risk factors are essentially represented by the MTHFR 677C > T homozygous or heterozygous genotype which results in impaired Hcy methylation to form methionine *(31)*. Nongenetic factors include older age *(28)*, male gender *(28)*, RA-specific features *(29)*, and disease-modifying antirheumatic

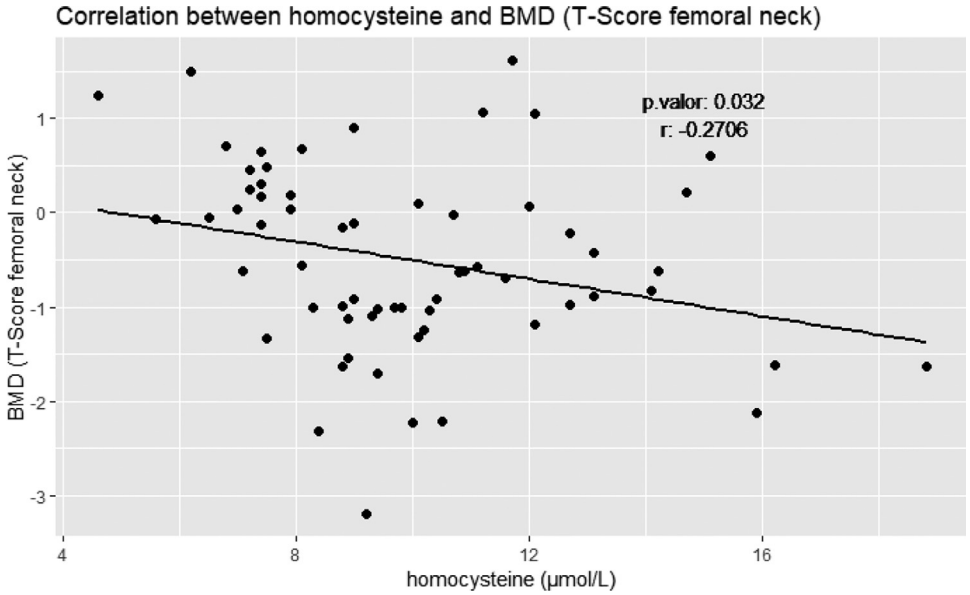

Fig. 2. Correlation between homocysteine and bone mineral density (BMD; T-score femoral neck).

Table 4
Correlations Between Homocysteine and Bone Mineral Density

	RA		HC	
	r	*p*	*r*	*p*
BMD (g/cm^2)				
Lumbar spine	0.02	0.883	−0.23	0.073
Femoral neck	−0.28	0.028	−0.19	0.153
Total femur	−0.26	0.046	−0.14	0.301
T-score				
Lumbar spine	−0.29	0.024	−0.22	0.085
Femoral neck	−0.27	0.032	−0.18	0.162
Total femur	−0.28	0.029	−0.12	0.339
Z-score				
Lumbar spine	−0.27	0.035	−0.16	0.222
Femoral neck	−0.25	0.052	−0.12	0.359
Total femur	−0.26	0.047	−0.07	0.603

Abbr: BMD, bone mineral density; HC, healthy control, RA, Rheumatoid Artrhitis.

drugs *(32)*. RA-specific features that influence the development of HHcy are mainly immunoinflammatory activation together with extra-articular features (e.g., gastrointestinal disorders and kidney dysfunction), and their resultant B vitamins (folic acid, pyridoxine, and cobalamine) deficiency *(33)*.

In the present study, we report for first time that high serum Hcy levels were inversely associated to lumbar spine BMD and femur neck BMD in patients with RA, and this association was independent of age. The association between HHcy and osteoporosis has previously been evaluated in the general population. Baines et al *(12)* showed a significant association of Hcy with BMD as well as significantly higher Hcy levels in the osteoporosis group. In the same way, Bozkurt et al *(9)* found that plasma Hcy levels were associated with osteoporosis in a sample of Turkish postmenopausal women. Bucciarelli et al *(11)* also showed a negative association between Hcy levels and BMD of total femur in a large cohort of postmenopausal women. Bahtiri et al *(27)* found that Hcy status, but not vitamin B12 status, is associated with BMD in a cohort of postmenopausal women. Similarly, Ouzzif et al *(26)* concluded that Hcy as well as vitamin B12 levels are independent risk factors for osteoporosis in Moroccan postmenopausal women. A meta-analysis of Zhang et al *(34)* concluded that Hcy and vitamin B12, but not folate, were related to BMD in postmenopausal osteoporosis. Roblin et al evaluated the association between HHcy and osteoporosis and low-bone mineralization in patients with Crohn's disease *(35)*. These authors observed that HHcy was strongly associated with low-bone mineralization and osteoporosis in Crohn's disease patients both at the hip and spine.

There were some limitations in our study that should be considered: this study was a cross-sectional analysis that reflected the status of a population in a particular period. The lack of data on folate and vitamin B12 status in these subjects made it impossible to analyze their potential influence in predicting BMD. Although a small sample size is a limitation of this study, power calculation indicated that the number of subjects was sufficient to establish statistical significance.

In conclusion, patients with RA have high levels of Hcy that correlate, inversely, with bone mass suggesting that HHcy is a risk factor for osteoporosis in patients with RA.

Table 5
Multiple Regression Analysis of BMD in Hip and Lumbar Spine With all Other Parameters in RA Patients

	Hip BMD (T-score)			Lumbar spine BMD (T-score)		
	β	95%CI	*p*	*B*	95%CI	*p*
Age	−0.045	(−0.07, −0.02)	0.001	−0.092	(−0.14, −0.05)	<0.001
Smoking	−0.004	(−0.51, 0.5)	0.987	−0.318	(−1.23, 0.6)	0.499
BMI	−0.062	(−0.16, 0.03)	0.214	−0.154	(−0.32, 0.02)	0.082
Body weight	0.040	(0, 0.08)	0.036	0.092	(0.03, 0.16)	0.007
Hcy	−0.084	(−0.16, −0.01)	0.030	−0.128	(−0.26, 0)	0.062
25-(OH)D	0.008	(−0.01, 0.03)	0.400	0.004	(−0.03, 0.04)	0.809
Intact PTH	−0.002	(−0.01, 0.01)	0.662	0.006	(−0.01, 0.02)	0.524
Prednisone	0.266	(−0.28, 0.81)	0.340	0.230	(−0.75, 1.21)	0.649

Abbr: 25-(OH)D, 25-hydroxy vitamin D; BMD, bone mineral density; BMI, body mass index, Hcy, homocysteine; PTH, parathyroid hormone; RA, rheumatoid arthritis.

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