Osteoprotegerin and osteocalcin are associated with atherosclerosis in patients with rheumatoid arthritis: a prospective cohort study

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Abstract

Objective

Patients with rheumatoid arthritis (RA) have an accelerated progression of atherosclerosis. The aim of this study was to examine the associations between subclinical atherosclerosis, assessed by intima-media thickness (IMT), and regulators of bone formation, markers of bone turnover and bone mineral density (BMD) in patients with RA.

Methods

Patients with new-onset RA (n=79), aged ≤60 years at diagnosis, were consecutively included in a study of development of atherosclerosis. Ultrasound measurement of IMT of the common carotid artery was undertaken at inclusion (T0) and after 11 years (T11) (n=54). Bone turnover biomarkers were examined in samples collected at T0 and T11. BMD was assessed at T11.

Results

In patients with RA, osteocalcin (OCN) and osteoprotegerin (OPG) measured at T11 were significantly associated with IMT at T11, adjusted for systolic blood pressure (SBP) and age. BMD at T11 and the bone turnover markers procollagen type 1 N-terminal propeptide (P1NP) and carboxy-terminal crosslinked C-terminal telopeptide (CTX) were not associated with IMT. OPG, OCN and sclerostin at T0 were significantly associated with IMT at T11, and OPG and OCN at T0 were associated with change in IMT from T0 to T11. The associations between IMT and bone biomarkers were stronger in patients with joint erosions at onset of RA, than in patients with non-erosive disease.

Conclusion

Atherosclerosis in patients with RA is associated with OPG and OCN, but not with BMD or markers reflecting ongoing bone turnover, indicating that atherosclerosis is not associated with bone turnover per se.

Key words

rheumatoid arthritis, atherosclerosis, osteoporosis, bone remodelling

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Introduction

Patients with rheumatoid arthritis (RA) have an increased morbidity and mortality due to cardiovascular disease (CVD), compared to the general population (1–3). This increase in CVD corresponds to a higher burden of atherosclerosis, measured as increased carotid intima-media thickness (IMT) (4, 5). The increased burden of atherosclerosis in patients with RA is not explained completely by traditional cardiovascular risk factors, nor by inflammation or other RA-specific factors (6). Osteoporosis and a reduced bone mineral density (BMD) are associated with an increased risk of cardiovascular disease and atherosclerosis in the general population (7, 8), and osteoporosis is more common in patients with RA compared with the general population (9). Studies in patients with RA have shown that decreased bone mineral density and increased arterial pulse-wave velocity are related (10), and that RA-patients with a fragility fracture have almost a doubled risk of a cardiovascular event, compared with patients without fractures (11).

Bone mineral density can be assessed using dual x-ray absorptiometry (DXA), a technique based on the different attenuation of x-rays in bone and soft tissue. Since the x-rays in DXA have two different energies with different attenuation from the bone, the bone mineral density can be quantified (12). The constant synthesis and degeneration of bone can be examined using markers of bone resorption and generation. A common marker of resorption is C-terminal cross-linked telopeptide (CTX), a collagen degradation product, whereas procollagen type I N-terminal propeptide (P1NP) is cleaved from collagen during bone formation. In addition to these markers of ongoing bone turnover, other biomarkers associated with bone can be examined, some of them supposed to be regulators of bone turnover, *e.g.* osteoprotegerin (OPG) and receptor-activator of nuclear factor κB ligand (RANKL) (13).

An individual's risk of osteoporotic fracture can be estimated using the computer based algorithm FRAX, where probability is calculated from age, sex, body mass index (BMI) and dichotomised risk factors comprising prior fragility fracture, parental history of hip fracture, current tobacco smoking, ever use of long-term oral glucocorticoid use, rheumatoid arthritis, other causes of secondary osteoporosis and high alcohol consumption (14). The output of FRAX is the 10-year probability of a major osteoporotic fracture (clinical spine, hip, humerus or wrist fracture).

Several bone biomarkers have been associated with atherosclerosis in the general population, *e.g.* OPG (15), RANKL (16), osteopontin (OPN) (15), dickkopf-1 (17), sclerostin (SCN) (17), and parathyroid hormone (PTH) (18). In patients with RA, bone markers have been less studied in the context of atherosclerosis, but OPG has been associated with coronary artery calcification (19), carotid plaque (20), intima-media thickness (IMT) and endothelial activation (21), pulse-wave velocity (22), and established cardiovascular disease (23).

The aim of this study was to examine the relations between atherosclerosis, assessed by intima-media thickness, and markers of bone turnover, regulators of bone turnover, estimated risk of fracture, and bone mineral density in patients with RA, compared with controls.

Methods

The present study is part of an ongoing regional prospective study of atherosclerosis in patients with RA, followed since the time of diagnosis. The procedures have been described in detail previously (24). All patients resident in the three northernmost counties of Sweden with new-onset RA, less than 60 years old, fulfilling the 1987 criteria for RA (25) and with symptoms of RA for less than one year at time of diagnosis, were invited to participate in a prospective study of atherosclerosis. Controls matched for age and sex were also included. The first examination soon after diagnosis is hereafter denominated T0 (n=79). At T0, 79 patients and 44 controls were examined, of whom 54 patients and 31 controls were re-assessed after eleven years (T11). The examinations were performed at the hospitals in Umeå, Sunderbyn and Östersund. Blood lipids were measured at T0 and

T11 using routine methods at the local hospitals where the patients were examined. Body weight and height were registered and BMI calculated. Data on cardiovascular risk factors are read from surveys and studies of patients´ records. Radiological examinations of hands, wrists and feet at T0 were examined by two trained rheumatologists and scored according to Larsen (26). Intima-media thickness was assessed by ultrasound examinations of the right common carotid artery, where IMT of the far wall was measured proximal to the bulb, as described previously (24). Blood pressure was measured at the ultrasound examination.

Biomarkers reflecting bone turnover were measured from frozen samples collected at T0 and T11, stored at -80° Celsius. Serum concentrations of osteopontin (OPN), osteoprotegerin (OPG), osteocalcin (OCN), sclerostin (SCN), dickkopf-related protein1 (DKK-1), and parathyroid hormone (PTH) were determined in samples using a multiplex assay (HBNMAG-51K-07, Millipore Corporation, Billerica, MA) according to manufacturer's protocol. Serum concentration of receptor activator of nuclear factor ϰB ligand (RANKL) was analysed using Human RANKL ELISA (BioVendor, Karasek, Czech Republic) according to the manufacturer´s protocol. This analysis measures both free RANKL and OPG-bound RANKL. Analysis of serum concentrations of P1NP, CTX, calcium, phosphate, and 25-OH-vitamin D was performed using chemiluminescense (P1NP and CTX), electrochemiluminescense (vitamin D) and photometric (calcium and phosphate) routine methods at the Department of clinical chemistry at Karolinska University hospital. Measurement of RANKL was undertaken in a non-selected subset of patients (n=24). Bone mineral density (BMD) was measured in patients and controls resident in Västerbotten, using dual-energy x-ray absorptiometry (DXA) (Lunar DPX-L, software version 1:3, Lunar, Madison, WI, USA). BMD for the spine was derived from the whole body composition scan using region of interest for the spine. In patients with RA, risk of fracture at diagnosis of RA was calculated using the Swedish version of FRAX without BMD included in the algorithm (14).

Table I. Demographic data and data on cardiovascular risk factors in 54 patients with RA and 31 controls. Numbers are mean (SD), except when indicated else.

Demographic	RA (n=54)	Controls (n=31)
Age, years	58 (10)	60 (11)
Female, n (%)	47 (87)	25 (81)
Cardiovascular risk factors		
Diabetes T0, n (%)	1 (2)	0
Diabetes T11, n (%)	2 (3.7)	1/30 (3.3)
Current smoking T0, n (%)	11 (20)	2 (6)
Ever smoking T11, n (%)	42 (78)	14 (45)
Antiresorptive treatment T11, n (%)	0	0
Calcium supplementation T11, n (%)	16 (30)	0
Vitamin D supplementation T11, n (%)	15 (28)	1 (3)
Systolic blood pressure at T0, mm Hg	122 (14)	118 (11)
Systolic blood pressure at T11, mm Hg	130 (14)	124 (11)
Cholesterol at T0, mmol/L	5.5 (0.9)	5.4 (1.1)
Cholesterol at T11, mmol/L	5.5 (1.1)	5.5 (1.2)
Statin treatment at T0, n (%)	1 (1.9)	0
Statin treatment at T11, n (%)	9/53 (17)	7 (23)
BMI at T11, kg/m^2	25.6 (4.7)	26.9 (4.9)
IMT at T0, mm	0.51 (0.12)	0.54 (0.12)
IMT at T11, mm	0.67 (0.16)	0.63 (0.13)
Rheumatoid arthritis		
ESR T11, mm/h, median (Q1-Q3)	13 (10-22)	NA
CRP T11, mg/L, median (Q1-Q3)	3.5 (0.9-9.0)	NA
Tender joint count T11, n, median (Q1-Q3)	0 (0-2)	NA
Swollen joint count T11, n, median (Q1-Q3)	0 (0-2)	NA
DAS28 T11	2.6 (1.4)	NA
HAQ T11, median (Q1-Q3)	0.13 (0-0.63)	NA
Positive anti-CCP, n (%)	35 (65)	NA
Methotrexate as first DMARD, n (%)	38/52 (73)	NA
Biologic DMARD T11, n (%)	15 (28)	NA
TNF-infibitor T11, n (%)	11 (20)	NA
Ever corticosteroid treatment T11, n (%)	43 (80)	1 (3)

Anti-CCP: positive anti-CCP-antibodies; BMI: body mass index; DAS28: Disease Activity Index of 28 joints; HAQ: Health Assessment Questionnaire; IMT: intima-media thickness; NA: not analysed; T0: examination at baseline; T11: Follow-up eleven years after baseline.

Statistics

Descriptive data is presented as mean (SD) or median (quartiles), depending on distribution. Analysis of differences between patients and controls was done with Mann-Whitney test or student's t-test, depending on distribution. Associations between explanatory variables and IMT was analysed using linear regression models. Variables with skewed distribution were log-transformed to reach normality before entering linear regression models. The multivariable regression models were constructed to explore the relation between the biomarkers, BMD, fracture risk, and IMT, and to what extent the observed IMT could be explained by the model. What variables to include in multivariable linear regression models was decided from the results of univariable regression models and clinical assumptions, but variables that lowered R^2 were excluded from the final models. Collinearity of independent variables was tested in multiple linear regression models, and a variable inflation factor of more than four was regarded intolerable. Since RANKL and BMD was not analysed in all patients, regression models including them both had limited degrees of freedom. p-values<0.05 were regarded statistically significant. Reported values of R^2 are adjusted R^2. All statistical analyses were performed using SPSS v. 25 (IBM SPSS INC, Chicago, Illinois, USA).

Results

Data on demography, cardiovascular risk factors and variables associated with RA in the 54 patients and 31 controls is presented in Table I. Of the 79 included patients at T0, one had died,

three had been found not to have RA, one suffered from severe dementia, one had moved, and 11 declined follow-up at T11. Furthermore, ultrasound examinations for measurement of IMT in seven patients was lost in a hardware crash. Among the controls, four had moved and nine declined follow-up. Data on bone markers and bone mineral density in the patients and controls are presented in Table II. Radiographs for Larsen score at T0 was present in 42 of the 54 patients.

Multivariable models at T11

OPG and OCN, measured at T11, were significantly associated with IMT at T11 in a linear regression model adjusted for age and systolic blood pressure (Table III). The relation with IMT was positive for OPG and negative for OCN. No other marker or regulator of bone turnover, or bone mineral density, were significantly associated with IMT at T11 or improved the explanation of variation in IMT when included in regression models. Linear regression models including the bone turnover markers P1NP and CTX measured at T11 did not perform better than regression models without those markers (Table IV). Of the traditional cardiovascular risk factors, only age and systolic blood pressure were significantly associated with IMT at T11. BMD, OPN, RANKL, SCN, PTH, or DKK-1 measured at T11 were not associated with IMT at T11 in linear regression models including all patients with RA (data not shown). No bone biomarker was significantly associated with IMT in controls, adjusted for age and systolic blood pressure (data not shown).

Predictive multivariable models at T0

In multivariable regression models on patients with RA, with IMT at T11 as dependent variable, the independent variables OCN, OPG and log SCN, all examined at T0, were associated with IMT at T11 (Table V). No other bone biomarker at T0 was associated with IMT at T11 in controls.

Patients with erosions

Linear regression models explaining IMT at T11, with independent variables OPG and OCN, had higher R^2 in patients with joint erosions at disease onset, than in patients without erosions. This was seen for models with OCN and OPG from both T0 and T11 (Table VI a-b). RANKL at T11 and IMT at T11 was highly associated in patients with erosions, as illustrated in Figure 1.

Change in IMT over time

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Table II. Levels of bone biomarkers and bone mineral density in 54 patients with RA and 31 controls, measured at T0 and T11. Numbers are mean (SD), except when indicated else.

	Patients n=54	Controls n=31	*p*-value
Osteopontin T0, ng/mL	20.2 (13.1)	16.1 (10.4) n=29	0.14
Osteopontin T11, ng/mL	22 (8.5)	19.4 (7.5)	0.12
Sclerostin T0, ng/mL, median (Q1-Q3)	2.4 (1.3-3.8) n=38	1.9 (1.2-5.1) n=20	0.8
Sclerostin T11, ng/mL, median (Q1-Q3)	3.0 (1.7-5.7) N=46	2.4 (1.4-3.6) n=26	0.07
Osteoprotegerin T0, pg/mL	345 (119)	407 (135) n=29	**0.03**
Osteoprotegerin T11, pg/mL	444 (260)	356 (96)	**0.03**
Osteocalcin T0, ng/mL	12.7 (4.8)	13.9 (6.1) n=28	0.5
Osteocalcin T11, ng/mL	14.1 (8.7)	14.1 (8.7)	0.9
RANKL T0, pmol/L, median (Q1-Q3)	728 (298-1128) n=36	NA	
RANKL T11, pmol/L, median (Q1-Q3)	565 (259-1044) n=24	NA	
P1NP T11, ng/mL, median (Q1-Q3)	59 (42-94)	58 (42-81)	0.2
CTX T11, ng/L	283 (232)	379 (218)	0.06
25-OH Vitamin D T11, nmol/L	76 (27)	75 (22)	0.8
Calcium T11, mmol/L	2.37 (0.078)	2.39 (0.10)	0.4
Phosphate T11, mmol/L	1.08 (0.18)	1.05 (0.15)	0.5
PTH T0, pg/mL, median (Q1-Q3)	83 (60-111)	152 (95-223) n=29	**0.001**
PTH T11, pg/mL, median (Q1-Q3)	88 (64-144)	89 (65-120)	0.4
DKK-1 T0, pg/mL	586 (231)	694 (330) n=29	0.12
DKK-1 T11, pg/mL	446 (201)	384 (131)	0.13
Bone mineral density, g/cm^2	1.02 (0.12) n=35	1.07 (0.13) n=28	0.18

CTX: C-terminal cross-linked telopeptide; DKK-1: dickkopf-related protein 1; NA: not analysed; P1NP: procollagen type I N-terminal propeptide; PTH: parathyroid hormone; RANKL: receptor activator of nuclear factor ϰB ligand; T0: examination at baseline; T11: Follow-up eleven years after baseline.

Table III. Multivariable linear regression models with IMT at T11 (1/10 mm) as response variable.

	Model 1	Model 2	Model 3
Variable	B (CI 95%)	B (CI 95%)	B (CI 95%)
BP T11, mm Hg		0.04 (0.01; 0.06)**	0.02 (-0.005; 0.04)
OCN T11, ng/mL	-0.09 (-0.2; -0.02)**	-0.09 (-0.1; -0.02)**	-0.05 (-0.1; -0.0001)*
OPG T11, ng/mL	3.7 (1.6; 5.8)**	3.4 (1.4; 5.4)**	2.0 (0.2; 3.8)*
Age, years			0.08 (0.05; 0.1)***
	R^2 0.16	**R^2 0.26**	**R^2 0.48**

B: unstandardised B; BP: systolic blood pressure; IMT: intima-media thickness; OCN: osteocalcin; OPG: osteoprotegerin; R^2: adjusted R^2; T11: follow-up eleven years from baseline.
95% CI: 95% confidence interval. *p<0.05, **p<0.01, ***p<0.001.

Table IV. Multivariable linear regression models with IMT at T11 (1/10 mm) as response variable.

	Model 1	Model 2	Model 3	Model 4
Variable	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)
BP T11, mm Hg	0.02 (-0.007; 0.04)	0.04 (0.01; 0.07)**	0.01 (-0.01; 0.04)	
Log P1NP T11, ng/mL		-0.3 (-1.5; 1.0)	-0.5 (-1.4; 0.4)	-0.2 (-1.5; 1.1)
Log CTX T11, ng/mL		-0.1 (-0.9; 1.2)	0.5 (-0.3; 1.3)	0.4 (-0.7; 1.5)
Age, years	0.1 (0.06; 0.13)***		0.1 (0.07; 0.14)***	
	R^2 0.45	**R^2 0.09**	**R^2 0.45**	**R^2 -0.03**

B: unstandardised B; BP: systolic blood pressure; CTX: C-terminal cross-linked telopeptide; IMT: intima-media thickness; P1NP: procollagen type I N-terminal propeptide; T11: follow-up eleven years from baseline. 95% CI: 95% confidence interval. *p<0.01, **p<0.001.

Table V. Multivariable linear regression models with IMT at T11 (1/10 mm) as response variable.

	Model 1	Model 2	Model 3	Model 4	Model 5
Variable	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)
BP T0, mm Hg		0.03 (0.007; 0.06)*		0.03 (0.004; 0.06)*	0.02 (-0.009; 0.04)
OCN T0, ng/mL	-0.08 (-0.16; 0.003)$^{p=0.06}$	-0.09 (-0.2; -0.02)*	-0.09 (-0.2; 0.01) $^{p=0.08}$	-0.1 (-0.2; -0.04)**	-0.06 (-0.1; 0.02)
OPG T0, ng/mL	5.5 (2.2; 8.8)**	2.6 (-0.4; 5.7)$^{p=0.09}$	5.8 (1.7; 10)**	1.4 (-2.1; 4.8)	1.0 (-2.1; 4.1)
Log SCN T0, ng/mL			-0.5 (-1.2; 2.2)	1.4 (0.02; 2.7)*	1.2 (0.02; 2.4)*
Age, years					0.06 (0.02; 0.1)**
	R^2 0.19	**R^2 0.24**	**R^2 0.23**	**R^2 0.39**	**R^2 0.52**

B: unstandardised B; BP: systolic blood pressure; IMT: intima-media thickness; OCN: osteocalcin; OPG: osteoprotegerin; SCN: sclerostin; T0: baseline; T11: follow-up eleven years from baseline. 95% CI: 95% confidence interval. *p<0.05, **p<0.01.

Table VIa. Multivariable linear regression models with independent variables from T0 and response variable IMT at T11 (1/10 mm) in the 42 patients who had radiographs at baseline.

	Patients with erosions at baseline. n=14			Patients without erosions at baseline. n=28		
	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)
BP T0, mm Hg	0.06 (0.01; 0.1)*		0.04 (-0.02; 0.1)	0.02 (-0.02; 0.06)		0.03 (-0.02; 0.07)
OCN T0, ng/mL		-0.2 (-0.3; -0.001)*	-0.1 (-0.3; 0.04)		-0.08 (-0.2; 0.06)	-0.07 (-0.2; 0.05)
OPG T0, ng/mL		5.2 (-0.4; 10.8) $^{p=0.07}$	3.1 (-3.1; 9.2)		8.9 (2.4; 15)**	0.5 (-6.8; 7.8)
	R^2 0,33	**R^2 0.32**	**R^2 0.40**	**R^2 0.02**	**R^2 0.18**	**R^2 -0.006**

B: unstandardised B; BP: systolic blood pressure; IMT: intima-media thickness; OCN: osteocalcin; OPG: osteoprotegerin; T0: baseline; T11: follow-up eleven years from baseline. 95% CI: 95% confidence interval. *p<0.05, **p<0.01.

Table VIb. Multivariable linear regression models with independent variables from T11 and response variable IMT at T11 (1/10 mm).

	Patients with erosions at baseline. n= 14			Patients without erosions at baseline. n=28		
	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)	B (CI 95%)
BP T11, mm Hg	0.07 (-0.02; 0.16)		0.05 (-0.02; 0.12)	0.05 (0.01; 0.09)		0.05 (0.006; 0.09)*
OCN T11, ng/mL		-0.2 (-0.4; -0.009)*	-0.17 (-0.35; 0.02)$^{p=0.07}$		-0.05 (-0.1; 0.04)	-0.04 (-0.1; 0.04)
OPG T11, ng/mL		9.6 (3.7; 15.5)**	8.8 (3.1; 14.5)**		2.5 (-0.6; 5.5)	1.8 (-1.0; 4.6)
	R^2 0.13	**R^2 0.45**	**R^2 0.52**	**R^2 0.19**	**R^2 0.03**	**R^2 0.18**

B: unstandardised B; BP: systolic blood pressure; IMT: intima-media thickness; OCN: osteocalcin; OPG: osteoprotegerin; T0: baseline; T11: follow-up eleven years from baseline. 95% CI: 95% confidence interval. *p<0.05, **p<0.01.

IMT from T0 to T11 as dependent variable, OPG (β 2.0, p=0.07) and OCN (β -0.07, p=0.02) at T0 were associated with increase in IMT in patients with RA, but not in controls. R^2 for the model was 0.12. In another model in patients with RA where T0 variables OPG, OCN, systolic blood pressure and age were included, no variable was statistically significant and R^2 still 0.12. No other bone biomarker analysed at T0 was associated with change in IMT from T0 to T11 (data not shown). Neither in patients, nor in controls, was change in PTH, OPG, OCN, SCN, RANKL, OPN, or DKK-1 from T0 until T11 associated with change in IMT from T0 to T11, or with IMT at T11 (data not shown).

Relation between inflammation and bone markers

In patients with RA, logESR at T11 was significantly related to OPG at T11 (standardised β 0.35, p=0.02) and RANKL at T11 (standardised β 0.45, p=0.03). However, those two biomarkers were not associated with swollen or tender joint counts, HAQ, DAS28 or logCRP. No other bone biomarker, levels of vitamin D, estimated fracture risk, or BMD was associated with any measures of inflammation, neither at T0 nor T11 (data not shown).

Pharmacological treatment

Supplementation with calcium or vitamin D at T11 or treatment with corticosteroids was not associated with neither IMT nor levels of regulators or markers of bone formation, nor was treatment with corticosteroids (Supplementary Fig. S1). Treatment with TNF inhibitors at T11 was associated with level of sclerostin at T11, but not with other biomarkers reflecting bone formation, nor IMT.

Fracture risk

The estimated 10-year risk of an osteoporotic fracture at T0, was significantly associated with IMT at T0 and T11 and change in IMT between the two measurements. Since age and most other traditional risk factors except blood pressure are included in FRAX, adjustment for those risk factors could not be done in regression models including fracture

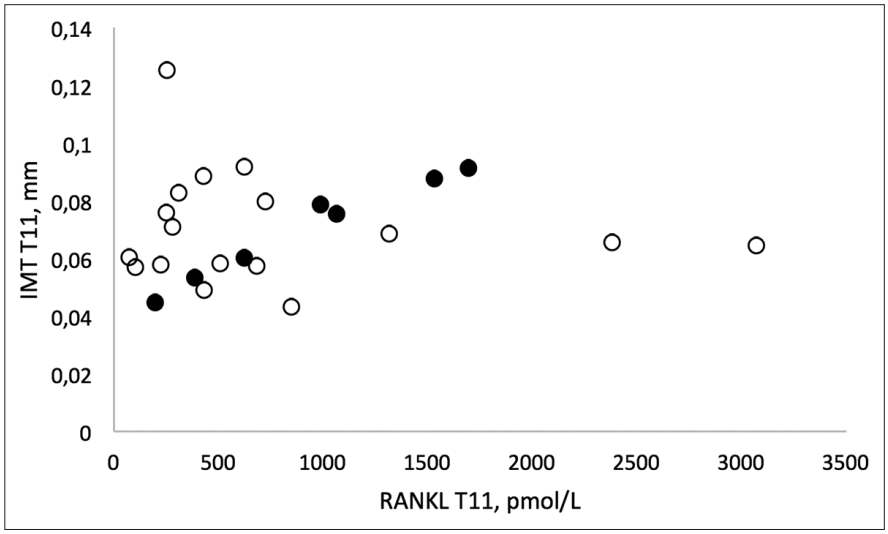

Fig. 1. Scatter plot of IMT at T11 and RANKL at T11. Filled circles denote patients with erosions at T0, open circles patients without erosions at T0. Standardised β=0.98, $p=3x10^{-5}$ for RANKL at T11 in a univariable regression model with IMT at T11 as dependent variable in patients with erosions at T0. IMT: intima-media thickness; RANKL: receptor activator of nuclear factor κB ligand.

risk. However, *p*-values were higher and R^2 lower than in corresponding models where IMT was analysed with age as independent variable (data not shown). Bone mineral density at T11 was not associated with estimated fracture risk at T0. Fracture risk was not estimated in controls.

Discussion

In this prospective longitudinal study, IMT was strongly associated with some of the markers of bone turnover in patients with RA. Eleven years after diagnosis of RA, OPG and OCN were significantly associated with IMT, adjusted for traditional cardiovascular risk factors, and OPG, OCN and SCN measured when the patients were newly diagnosed with RA were predictors of IMT at follow-up eleven years later. However, this should not be interpreted as an indication of an association between IMT and bone turnover. In fact, the collagen-derived biomarkers P1NP and CTX, being direct measures of bone turnover, were not associated with IMT, although this would have been likely if increased IMT was a direct consequence of increased bone turnover. Likewise, BMD at T11 was not associated with IMT in our study. It is also worth noting that RANKL, an important stimulator of osteoclast activity (27), was not associated with IMT, whereas the RANKL inhibitor OPG was. This implies that the mechanisms behind the association between regulators of bone turnover and atherosclerosis are independent from bone turnover *per se*.

OPG at T11 was associated with IMT at T11 in several multiple regression models, adjusted for traditional cardiovascular risk factors. This is in line with previous cross-sectional studies of patients with RA, where OPG has been associated with coronary artery calcification (19), carotid plaques (20), IMT (21, 22), markers of endothelial activation (21), pulse-wave velocity (22), and established cardiovascular disease (23). Our study is prospective: OPG and IMT were measured both soon after diagnosis of RA and after eleven years of disease, facilitating the possibility to study the associations over time. We found that the level of OPG both at T0 and T11 was associated with IMT at T11, and OPG at T0 was associated with change in IMT from T0 to T11, although the associations were not statistically significant in all models. However, change in levels of OPG from T0 to T11, was not associated with change in IMT from T0 until T11, a relation that would have been plausible if OPG itself caused atherosclerosis. This might be explained by the fact that OPG is produced in several tissues, including activated endothelial cells (28) and vascular smooth muscle cells (16), making OPG a potential marker of atherosclerosis, rather than causing atherosclerosis. In our results, OPG was positively associated with ESR, but not with other measures of inflammation in patients with RA, making it unlikely to be the missing link between inflammation and atherosclerosis. Neither a recent meta-analysis found a statistically significant relation between inflammation and OPG in patients with RA (29).

OCN had a negative association with both IMT at T11 and increase in IMT from T0 to T11. OCN is a somewhat peculiar molecule: it is the most abundant non-collagenous peptide found in the bone matrix, but also has several hormonal properties, including effects on glucose metabolism (30). Initially OCN was supposed to be produced by osteoblasts only, but has later been found to be produced also in the arterial wall (31). The results in previous studies of OCN in relation to atherosclerosis have been inconsistent, according to a recent meta-analysis (30). When it comes to IMT, both positive and negative relations with IMT have been identified, but the studies have been carried out in heterogenous populations, making comparisons difficult. However, morphological studies have consistently reported elevated levels of OCN in calcified atherosclerotic lesions (30). In our results, OCN at T0 is a negative predictor of change in IMT from T0 to T11, and OCN at T11 has a negative association with IMT at T11, indicating that OCN in patients with RA is associated with less pronounced atherogenesis.

Previous studies in patients with RA have identified a positive effect of anti-inflammatory treatment, in particular biological DMARD, on osteoporosis and markers of bone turnover (32, 33). In our results, those relations were not very prominent (Suppl. Fig. 2). This could be explained by heterogeneity in the treatment, since the patients during the eleven years of follow-up were treated according to clinical practice, and not by a treatment protocol. In this routine clinical setting, associations between treatments and other vari-

ables are also prone to be confounded by indication. The patients treated with biological DMARD or corticosteroids in our study do probably have a more aggressive RA, attenuating the relations between treatment and bone turnover.

A higher estimated fracture risk at diagnosis of RA was in our study associated with increased IMT in patients with RA. However, age and other traditional cardiovascular risk factors are included in the FRAX algorithm used for estimation of fracture risk, making this association very likely, and regression models involving FRAX were inferior to models where FRAX was substituted for age.

OPG and OCN at T0 were more predictive of IMT at T11 in patients with joint erosions, than in patients without erosions. A strong relation to IMT in these patients was also seen for OPG and OCN at T11. Furthermore, RANKL had an almost linear relation with IMT in the few patients with erosions where RANKL was analysed. Thus, erosive RA seems to be a marker of a disease phenotype where OGP, OCN and RANKL are related to IMT, but the bone turnover markers CTX and P1NP still were not associated with IMT in patients with erosions. This contradiction points out the need of further studies on how bone turnover and atherosclerosis are intertwined in patients with RA.

This study has some limitations. The first is the relatively small number of patients, and the even smaller number of controls. Another limitation is the fact that the blood sampling for bone markers was not performed in a standardised time of the day. This might have caused variation due to circadian rhythm of some of the biomarkers studied (OCN, CTX, P1NP, PTH, FGF-23, SCN). The blood samples were also stored for several years before analysis, in particular the baseline samples, which might cause variation in the analytes. OCN is present in two types, the carboxylated and the undercarboxylated, that have separate properties, but are not separated by our method of measurement. Furthermore, the measurement of BMD was derived from the whole body composition scan, without the possibility to exclude vertebrae with excessive spondylosis or compression.

On the other hand, there are strengths of the study. The first to mention, is the prospective design, that allows for analysis over time. Furthermore, the cohort is well characterised and unselected otherwise than by geography and age at inclusion (<60 years), and all patients were included soon after diagnosis of RA, making the cohort representative and homogenous in terms of duration of disease. Every ultrasound examination has been performed by the same experienced operator both at T0 and T11.

In conclusion, our study shows that levels of some bone biomarkers, in particular osteoprotegerin and osteocalcin, are related to IMT in patients with RA, especially in patients with joint erosions. However, the collagen derived markers of ongoing bone turnover P1NP and CTX are not associated with IMT, contradicting an association between atherosclerosis and bone turnover *per se*. This indicates that bone turnover and atherosclerosis have independent pathogenetic mechanisms.

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