Circulating Osteoprotegerin and Soluble RANK Ligand in Systemic Sclerosis

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ABSTRACT. ***Objective.*** Microvascular damage is an early pathogenetic event in systemic sclerosis (SSc). The receptor activator of nuclear factor-κB ligand (RANKL)/RANK/osteoprotegerin (OPG) system is involved in vascular biology. Our aim was to assess OPG and soluble RANKL (sRANKL) serum levels in patients with SSc and healthy controls.

Methods. Sixty patients with SSc (median age 58, range 31–72 yrs) and 60 healthy subjects matched for age, sex, and menopausal status were recruited. Serum OPG, sRANKL, soluble vascular cell adhesion molecule (sVCAM; marker of endothelial activation/injury), and bone turnover markers were measured. Bone mineral density in patients was assessed and cardiovascular/coronary risk was estimated.

Results. OPG was similar in the 2 groups, while sRANKL and sRANKL/OPG ratio was higher in patients ($p = 0.01$ for both). sVCAM was markedly higher in patients ($p < 0.001$). OPG levels correlated positively with age in both patients (Spearman $R = 0.50$, $p < 0.001$) and controls ($R = 0.56$, $p < 0.001$). In patients, OPG was lower in men and higher in those with active ulcers or calcinosis. sRANKL levels were higher in patients treated with platelet aggregation inhibitors, and correlated negatively with densitometric measures. 25-hydroxyvitamin D levels were significantly lower in patients ($p < 0.001$). In patients, OPG levels correlated positively with cardiovascular and coronary risk ($R = 0.28$, $p = 0.05$ and $R = 0.34$, $p < 0.01$, respectively) and were higher in patients with hypertension and left ventricular hypertrophy. sVCAM levels correlated positively with cardiovascular and coronary risk ($R = 0.27$, $p = 0.06$, and $R = 0.38$, $p < 0.01$, respectively).

Conclusion. Higher sRANKL levels and sRANKL/OPG ratio in patients with SSc are likely to be a consequence of altered bone microenvironment. We show a dissociation between the well established marker of endothelial activation/injury, sVCAM, and the alleged marker of vascular damage, OPG, in patients with SSc. Further studies are needed to better ascertain the relationships of the RANKL/RANK/OPG system with the progression of macro- and microvascular damage. (First Release Oct 1 2008; J Rheumatol 2008;35:2206–13; doi:10.3899/jrheum.080192)

Key Indexing Terms:
OSTEOPROTEGERIN SYSTEMIC SCLEROSIS BONE TURNOVER MARKERS
SOLUBLE RECEPTOR ACTIVATOR OF NUCLEAR FACTOR-κB LIGAND
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BONE MINERAL DENSITY CARDIOVASCULAR RISK

Systemic sclerosis (SSc) is a chronic connective tissue disease characterized by vascular damage and varying degrees of fibrosis of the skin and visceral organs. Endothelial injury, immune activation, and collagen deposition by activated fibroblasts are involved in the pathogenesis of the disease. Pathological appearances at the vascular level include the coexistence of features of endothelial cell (EC) activation and damage, intercellular gaps, recruitment of inflammatory cells, thrombosis, intimal proliferation, and adventitial fibrosis, with eventual vessel obliteration[1-4]. It is agreed that the microvasculature is primarily affected. Large-vessel disease may also occur[5-7].

In the last decade the new signaling system consisting of receptor activator of nuclear factor-κB ligand (RANKL), its receptor RANK, and its decoy receptor osteoprotegerin (OPG) has been intensively investigated. In the bone microenvironment, RANKL is expressed mainly by cells of the stromal-osteoblastic lineage; its binding to RANK stimulates the differentiation and fusion of osteoclast precursors, activates mature osteoclasts, and prolongs their lifespan by inhibiting apoptosis, thus enhancing bone resorption. OPG

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binds to RANKL, thus preventing RANKL-RANK interaction and inhibiting bone resorption[8]. In the immune system, RANKL-RANK interaction plays a crucial role in the biology of dendritic cells, promoting their survival and ability to stimulate T cells[9].

With regard to vascular biology, RANKL is virtually absent in normal vasculature but is overexpressed in vulnerable atherosclerotic lesions prone to rupture, and has been implicated in plaque destabilization because of its ability to promote matrix degradation, monocyte/macrophage chemotaxis, and vascular calcification[10,11]. Indeed, RANKL stimulates production of a variety of chemotactic and growth factors from EC and peripheral blood mononuclear cells. Moreover, in vascular smooth muscle cells it enhances matrix metalloproteinase activity and promotes osteogenic differentiation and calcification[10,11]. Recently, serum soluble RANKL (sRANKL) has emerged as a highly significant and independent risk predictor of cardiovascular events in a large-scale, prospective, and population-based survey[12].

OPG is highly expressed in human arterial walls, with aortic smooth muscle cells producing 20–30 times more OPG than EC[13]. In addition to preventing RANKL-RANK interaction, OPG may act in vascular pathophysiology by preventing interaction of tumor necrosis factor-related apoptosis-inducing ligand with its receptors and by its ability to bind syndecan-1, von Willebrand factor, and thrombospondin-1[13,14]. *In vitro*, OPG promotes endothelial cell survival, formation of endothelial cord-like structures, and leukocyte adhesion. OPG knockout mice show calcification of the aorta and renal arteries[13]. In the LDLR–/– mouse model of atherosclerosis, OPG administration significantly reduced the calcified lesion area without affecting atherosclerotic lesion size or number[15]. In humans, serum OPG levels are increased in patients with acute myocardial infarction, coronary artery disease, coronary and aortic calcifications, and increased carotid intima-media thickness[16]. Not surprisingly, they have been found to be associated with a number of established cardiovascular risk factors (including age, diabetes, hypertension, smoking, markers of systemic inflammation, chronic infection, and hyperhomocysteinemia)[16] and to predict cardiovascular mortality in elderly women and cardiovascular events in the general population[17,18]. Moreover, OPG expression is increased in the arterial media of diabetic patients[13,19], and OPG serum levels correlate negatively with flow-mediated vasodilation in healthy postmenopausal women[20] and type 1 and 2 diabetic[21,22] and hypothyroid[23] patients. Since *in vitro* studies and *in vivo* OPG knockout models are consistent with a protective action of OPG on EC, the supranormal levels found in patients with endothelial dysfunction and atherosclerosis have been attributed to inadequate compensatory response; OPG has been viewed as an inhibitor of calcification and a marker (rather than a mediator) of endothelial dysfunction and atherosclerosis[15]. However, a pathogenetic role through its effects on leukocyte adhesion and coagulation cannot be excluded[13].

To our knowledge, no previous study has examined the role of the RANKL/RANK/OPG system in SSc. The aims of our study were to compare OPG and sRANKL serum levels and sRANKL/OPG ratio in SSc patients and healthy subjects matched for sex, age, and menopausal status, and to search for correlations with estimated cardiovascular/coronary risk, an established marker of vascular damage (soluble vascular cell adhesion molecule, sVCAM), bone mineral density (BMD), and turnover markers.

MATERIALS AND METHODS

Study population. Sixty consecutive adult patients with SSc [48 women, 12 men; median age 58 yrs (range 31–72)] referred to the Rheumatology Unit of the Internal Medicine Department between 2002 and 2008 were recruited. The control group was composed of 60 healthy subjects [48 women, 12 men; median age 58 yrs (range 33–73)] matched for sex, age, and menopausal status, recruited in the same period at the Turin section of AVIS (Italian Blood Donors Association) among blood donors (up to 70 yrs of age) and former blood donors (the 6 subjects older than 70 yrs). Persons with autoimmune disease, history of any cardiovascular event, or taking chronic glucocorticoid treatment are not admitted for blood donation; donors taking drugs known to interfere with bone metabolism were excluded from our study.

SSc was diagnosed and classified according to the criteria proposed by the American College of Rheumatology[24] and LeRoy, *et al*[25]. Exclusion criteria were anamnestic history of diseases affecting bone; renal failure, defined as serum creatinine > 1.3 mg/dl and/or blood creatinine clearance < 60 ml/min, and/or history of scleroderma renal crisis; acute myocardial infarction or cerebral stroke; and malignancy.

Patients underwent examination, routine laboratory evaluation, chest radiograph, high-resolution computed tomography (HRCT) of chest, pulmonary function tests (PFT), electrocardiography (ECG), Doppler echocardiography, and capillaroscopy. They were evaluated for the presence of organ involvement: (1) gastrointestinal involvement was defined as any of the following: symptoms and/or signs of gastroesophageal reflux disease, esophageal hypomotility (on barium radiography), delayed gastric emptying (on barium radiography), malabsorption; (2) pulmonary involvement was defined as HRCT signs of interstitial lung disease and/or forced vital capacity (on PFT) and/or carbon monoxide diffusion factor < 80% of predicted values; pulmonary artery hypertension was defined by right-heart catheterization, which was performed in only 2 patients on the basis of echocardiographic finding of pulmonary arterial pressure > 35 mm Hg at rest and unexplained dyspnea; (3) heart involvement was defined as any of the following: symptomatic pericarditis, clinical evidence of left ventricular congestive heart failure, left ventricular diastolic dysfunction (on Doppler echocardiography), or arrhythmias or conduction disturbance (on electrocardiogram); (4) skeletal muscle involvement was defined as proximal muscle weakness and elevated serum creatine kinase level; (5) joint involvement was defined as inflammatory polyarthralgia or arthritis; (6) calcinosis was assessed by examination.

Study protocol was prepared according to the Declaration of Helsinki and subsequent relevant integrations, and approval by the local Ethical Committee was obtained. All participants gave written informed consent before participation.

Biochemical measurements. Blood samples were drawn at 8:00 AM, after overnight fast, from an antecubital vein, were centrifuged for 15 min at 1000 g within 2 h of collection, and immediately frozen at –20°C before analysis. The main analytes (sRANKL and OPG) were assayed in 2 runs: in 2007 (42 patients and respective controls) and 2008 (18 patients and respective controls). Limited data are available on the effects of storage on

circulating levels of sRANKL and OPG. Chan, *et al*[26] have reported significant decreases of sRANKL and OPG concentrations in plasma samples after 6 weeks and 6 months, respectively, of storage at –20°/–70°C; however, stability of serum samples was not assessed. Stern, *et al*[27] have reported no associations between storage duration and OPG or sRANKL concentrations in 139 serum samples assayed after 5–16 years of storage at –70°C, and no differences of means and variances for OPG and sRANKL concentrations between samples collected at 2 different visits 11 years apart.

Serum calcium, phosphorus, and albumin were measured by Aeroset System (Abbott Laboratories, Abbott Park, IL, USA), routinely used in the hospital laboratory. Serum calcium levels were always corrected for serum albumin as follows: corrected calcium (mmol/l) = total calcium (mmol/l) + 0.02[40-albumin (g/l)]. Serum OPG was measured by an ELISA developed in our laboratory using commercial reagents (OPG DuoSet, R&D Systems, Abingdon, UK), as described[28]. Capture antibody was a monoclonal mouse anti-human OPG; detection antibody was a biotinylated goat polyclonal anti-human OPG. Reagent diluent was phosphate buffered saline with 50% fetal calf serum and 1% bovine serum albumin. Serum total sRANKL, sVCAM, osteocalcin (OC), and C-telopeptide of type I collagen (CTX) were measured by commercial ELISA kits (total sRANKL: Immundiagnostik, Bensheim, Germany; sVCAM: R&D Systems; OC, CTX: Nordic Bioscience Diagnostics A/S, Herlev, Denmark). For sRANKL measurement, all samples were diluted 1:10 according to manufacturer's instructions. Serum 25-hydroxyvitamin D [25(OH)D] and bone-specific alkaline phosphatase (bALP) were measured by radioimmunoassay (Immunodiagnostic Systems, Boldon, UK, and Beckman Coulter, Fullerton, CA, USA, respectively).

Dynamic ranges, mdc, and intra- and inter-assay coefficients of variation (CV) were as follows: OPG: 62.5–4000 pg/ml, < 30 pg/ml, < 5% and 13.5%; sRANKL: 133–3600 pg/ml, < 50 pg/ml, < 5% and < 10%; sVCAM: 6.25–400 ng/ml, 1.6 ng/ml, 2.2% and 0.7%; OC: 6.3–100 ng/ml, 0.5 ng/ml, 2.6% and 4.7%; CTX: 148–2365 pg/ml, 12 pg/ml, 5.2% and 6.7%; bone ALP: 15–120 ng/ml, 2 ng/ml, 4.9% and 7.4%; 25(OH)D: 1.6–160 ng/ml, 1.2 ng/ml, 5.5% and 7.9%.

Cardiovascular risk. In patients, cardiovascular risk was estimated by 2 different algorithms, as reported[29]. The first was developed by the Italian National Institute of Health and applies to subjects aged 35–69 years to estimate 10-year absolute global cardiovascular (not only coronary) risk based on age, sex, blood pressure, total and high density lipoprotein (HDL)-cholesterol, smoking status, and diabetes. The second was developed by the Italian Society for the Study of Atherosclerosis and applies to subjects aged 30–74 years to estimate 10-year absolute coronary risk based on age, sex, blood pressure, total and HDL-cholesterol, smoking status, diabetes, and left ventricular hypertrophy. The 2 estimates were found to correlate at the highest degree of significance (R = 0.88, p < 0.0001). Data were available to estimate absolute cardiovascular risk also in a subgroup of 38 control subjects.

Dual-energy x-ray absorptiometry (DEXA). Bone densitometry was performed in 52 patients by DEXA using the Hologic QDR 4500 W instrument (Hologic, Waltham, MA, USA; software version 9.03), with longterm CV of 0.5% at the spine (assessed by the Hologic anthropometric spine phantom), and short-term *in vivo* CV of 1% and 1.5% for the lumbar spine (L1–L4) and total hip, respectively. Absolute BMD values (g/cm^2), T- and Z-scores, referred to the manufacturer's normative data for lumbar spine and to the National Health and Nutrition Examination Survey III dataset for the hip[30], were analyzed. The classical World Health Organization (WHO) criteria were used to define the conditions of normality, osteopenia, and osteoporosis (T-score > –1, between –1 and –2.5, or < –2.5, respectively)[31].

Statistical analysis. Database management and all statistical analyses were performed by Statistica 6.0 (Statsoft Inc., Tulsa, OK, USA). Normality of data was assessed by the Wilk-Shapiro test. Since most continuous variables showed non-normal distribution, data are presented as median (range), and differences and correlations were analyzed by 2-tailed Mann-Whitney U-test and Spearman R coefficient, respectively. Level of statistical significance was set at p < 0.05.

RESULTS

Demographic, clinical, densitometric, and laboratory data of patients are reported in Table 1. In patients, serum sRANKL and sRANKL/OPG ratio was significantly higher than in controls; no significant difference was observed in OPG levels; and serum sVCAM was markedly higher in patients (Figure 1). To exclude that differences were due to effects of longterm storage, we separately evaluated a subgroup of 16 patients and controls in whom blood samples were collected in April-May 2008. Relevant blood samples were assayed within 2 months from collection. As in the whole population, in patients we found higher sRANKL [39 (range 9–317) vs 8 (4–294) ng/ml; p < 0.01] and higher sVCAM levels [959 (649–1561) vs 654 (520–889) ng/ml; p < 0.001], while OPG levels were comparable to controls.

Table 1. Characteristics of patients with systemic sclerosis.

Age, yrs (range)	58 (31–72)
Men/women	12/48
Menopausal (%)	37 (77)
Menopausal age, yrs	48 (39–54)
Body mass index, kg/m^2	25.0 (15.4–37.1)
Former smokers/current smokers (%)	10 (17)/2 (3)
Disease duration from diagnosis, yrs	3 (0–39)
	n (%)
Diffuse SSc/limited SSc	13/47 (22/78)
Anti-topoisomerase I/anticentromere antibodies	24/24 (40/40)
Active ulcers	17 (28)
Raynaud's phenomenon	59 (98)
Gastrointestinal involvement	42 (70)
Lung involvement	32 (53)
Echocardiographic pulmonary artery pressure > 35 mm Hg	34 (57)
Heart involvement	8 (19)
Skeletal muscle involvement	8 (13)
Joint involvement	14 (23)
Calcinosis	5 (8)
Current therapy	
Low-dose prednisone (≤ 10 mg/day)	23 (38)
Immunosuppressant (cyclophosphamide/azathioprine)	9 (15)
Platelet aggregation inhibitor	44 (73)
Iloprost	20 (33)
Calcium+vitamin D	14 (23)
Bisphosphonates	7 (12)
	Median (range)
Lumbar spine BMD, g/cm^2	0.920 (0.593–1.140)
Lumbar spine T-score L1–L4	–1.450 (–4.130–0.840)
Lumbar spine Z-score L1–L4	–0.470 (–2.610–2.270)
Total hip BMD, g/cm^2	0.850 (0.543–1.088)
Total hip T-score	–0.820 (–3.270–1.130)
Total hip Z-score	–0.340 (–2.269–3.000)
25-hydroxyvitamin D, ng/ml	23 (3–92)
Serum corrected calcium, mmol/l	2.34 (1.89–2.60)
Serum phosphorus, mg/dl	3.7 (2.7–5.1)
Serum bALP, ng/ml	9.3 (1.0–27.0)
Serum OC, ng/ml	15.2 (0.3–42.5)
Serum CTX, ng/ml	0.78 (0.15–2.60)

Values are number of patients (%) or median (range). BMD: bone mineral density; bALP: bone-specific alkaline phosphatase; OC: osteocalcin; CTX: C-telopeptide of type I collagen.

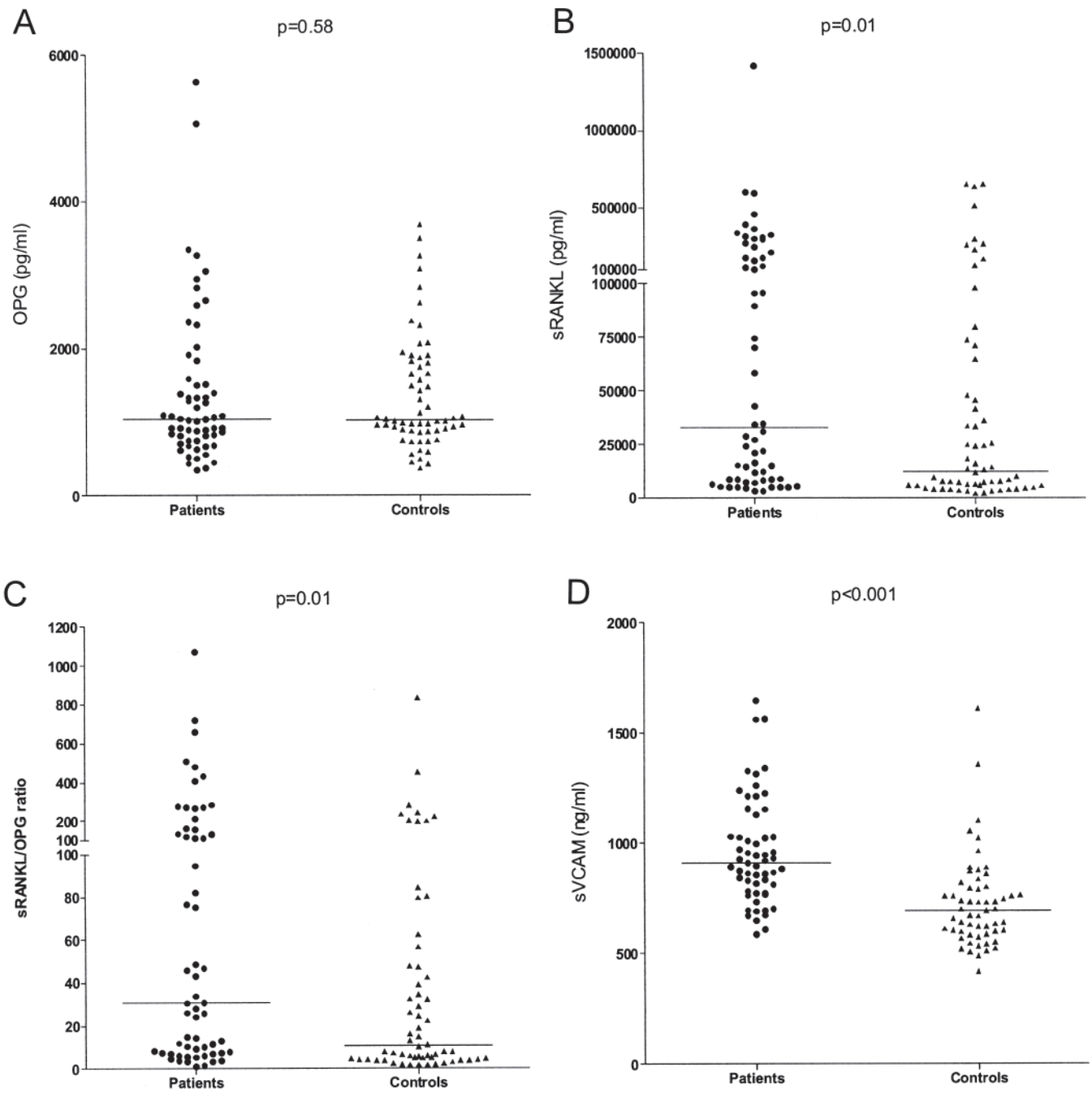

Figure 1. Serum levels of OPG (A) sRANKL (B), sRANKL/OPG ratio (C), and sVCAM level (D) in patients with SSc and controls. Individual data and median (horizontal line) are shown.

Subgroup and correlation analyses were performed to evaluate associations of the analytes with demographic, clinical, densitometric, and laboratory data listed in Table 1. When only women were considered, results were consistent with those obtained in the whole population; in contrast, lower OPG levels were found in male patients compared to both male controls and female patients (Table 2). OPG (but not sRANKL) levels correlated positively with age in both patients (Spearman R = 0.50, $p < 0.001$) and controls (R = 0.56, $p < 0.001$). In patients, OPG levels were significantly higher in those with active ulcers and those with calcinosis (Table 3). No differential pattern was noted in relation to drug treatment. For sRANKL, levels did not correlate with disease variables, but were significantly higher in patients taking platelet aggregation inhibitors. sVCAM was significantly higher in patients with heart involvement (Table 3), and did not correlate with either OPG or sRANKL.

Estimated 10-year absolute cardiovascular risk was simi-

Table 2. Serum OPG, sRANKL, and sVCAM levels and sRANKL/OPG ratio in patients with SSc and controls.

	Study Population			Men			Women		
	Patients, n = 60	Controls, n = 60	p	Patients, n = 12	Controls, n = 12	p	Patients, n = 48	Controls, n = 48	p
OPG, pg/ml	1045 (348–5627)	1026 (372–3680)	0.58	784 (348–1834)	1011 (593–3088)	0.06	1086 (374–5627)	1042 (372–3680)	0.78
sRANKL, ng/ml	33 (3–1421)	13 (2–651)	0.01	25 (5–211)	11 (3-265)	0.34	33 (3–1421)	13 (2–652)	0.02
sRANKL/OPG	31 (1–1072)	11 (2–835)	0.01	47 (8–409)	8 (2–283)	0.11	29 (1–1072)	12 (2–835)	0.04
sVCAM, ng/ml	907 (585–1648)	692 (417–1612)	< 0.001	946 (780–1343)	732 (520–1612)	0.01	888 (585–1648)	681 (417–1359)	< 0.001

sRANKL: soluble receptor activator of nuclear factor-κB ligand; OPG: osteoprotegerin; sVCAM: soluble vascular cell adhesion molecule. Data are reported as median (range). Comparisons between patients and controls were by Mann-Whitney U-test.

Table 3. Serum sRANKL, OPG, and sVCAM levels in patients with SSc: subgroup analyses.

		Patients With	Patients Without	p
OPG, pg/ml	Active ulcers	1518 (633–3356)	919 (348–5627)	0.04
	Calcinosis	2659 (919–5627)	1032 (348–5063)	0.03
	Hypertension	1308 (752–5627)	922 (348–3356)	0.04
	LVH	2663 (1336–5063)	923 (348–5627)	< 0.001
sRANKL, ng/ml	PAI	39 (5–1421)	15 (3–457)	0.03
sVCAM, ng/ml	Heart involvement	1010 (761–1563)	878 (585–1648)	0.03

Data are reported as median (range). Comparisons between subgroups were performed by Mann-Whitney U-test. sRANKL: soluble RANKL; OPG: osteoprotegerin; sVCAM: soluble VCAM; LVH: left ventricular hypertrophy; PAI: platelet aggregation inhibitors.

lar in a subgroup of 38 patients and respective controls (data not shown). In both patients and controls, OPG levels correlated positively with cardiovascular risk (R = 0.28, p = 0.05, and R = 0.40, p = 0.01, respectively); in patients they also correlated with coronary risk (R = 0.34, p < 0.01), and were higher in those with hypertension and left ventricular hypertrophy (Table 3). sVCAM levels correlated significantly with estimates of cardiovascular and coronary risk (R = 0.27, p = 0.06, and R = 0.38, p < 0.01, respectively), with no relationship with classical risk factors. Only 1 patient had diabetes, and 2 patients were current smokers, hence analysis of the effects of these factors was not performed. sRANKL did not correlate with any measure relevant to cardiovascular risk.

When DEXA results were considered, 14 patients were classified as osteoporotic and 16 as osteopenic according to WHO criteria. Densitometric measures of both lumbar spine and hip showed negative correlations with sRANKL but not OPG or bone turnover markers (Table 4). 25(OH)D levels were significantly lower in patients (Figure 2), even when only patients and controls matched for season of the year were compared (data not shown); 4 patients had a severe deficiency (< 10 ng/ml); 17 patients were in the insufficiency range (≥ 10 and < 20 ng/ml) and 17 in the mild hypovitaminosis D (20–30 ng/ml) range[32]. bALP and OC levels were similar in patients and controls (data not shown), while CTX was marginally higher in patients (Figure 2). Correlations of bone turnover markers are shown in Table 5; in patients (but not controls), OPG showed a negative correlation with CTX (R = –0.32, p = 0.01), while sRANKL correlated positively with OC (R = 0.33, p = 0.01).

In a multivariate regression analysis including active ulcers, calcinosis, coronary risk, and CTX as possible independent predictors of OPG (adjusted R^2 = 0.23, p < 0.001), the variables significantly associated with OPG were CTX (ß = –0.33, p < 0.01) and calcinosis (ß = 0.38, p < 0.01). In a multivariate regression analysis including platelet aggregation inhibitor, lumbar and hip BMD, and OC as possible

Table 4. Correlations of serum sRANKL with bone densitometric measures in patients with SSc.

	Patients, n = 52		Male Patients, n = 11		Female Patients, n = 41	
	Spearman R	p	Spearman R	p	Spearman R	p
Lumbar spine BMD	–0.35	0.01	–0.57	0.07	–0.33	0.04
Lumbar spine T-score	–0.26	0.06	–0.41	0.20	–0.26	0.10
Lumbar spine Z-score	–0.29	0.04	–0.18	0.59	–0.36	0.02
Total hip BMD	–0.34	0.02	–0.60	0.09	–0.32	0.05
Total hip T-score	–0.32	0.03	–0.68	0.04	–0.27	0.10
Total hip Z-score	–0.28	0.06	–0.70	0.04	–0.23	0.16

BMD: bone mineral density.

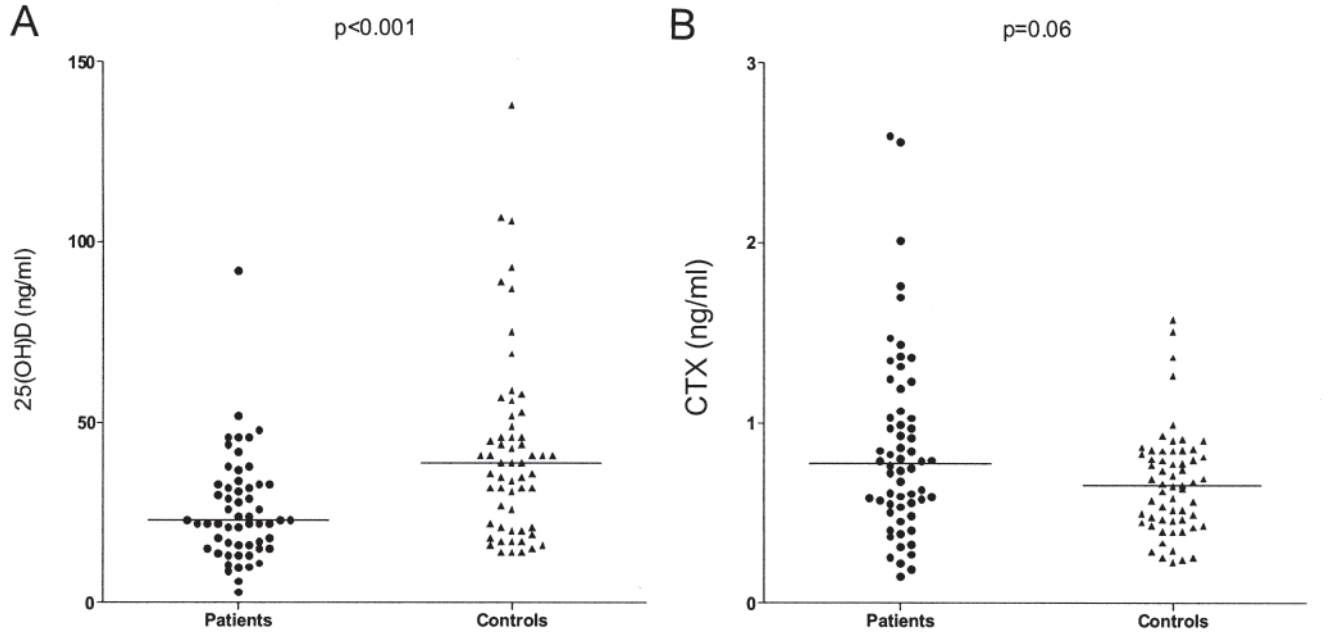

Figure 2. Serum levels of 25(OH)D (A) and CTX (B) in patients with SSc and controls. Individual data and median (horizontal line) are shown.

Table 5. Spearman R coefficients for correlations of serum sRANKL, OPG, 25(OH)D, and bone turnover markers in patients with SSc.

	sRANKL	bALP	OC	CTX	25(OH)D
OPG	0.12	0.21	–0.11	–0.32*	–0.002
sRANKL		0.13	0.33*	0.14	–0.06
bALP			0.44**	0.14	–0.22
OC				0.54**	–0.30*
CTX					–0.30*

sRANKL: soluble RANKL; OPG: osteoprotegerin; bALP: bone-specific alkaline phosphatase; OC: osteocalcin; CTX: C-telopeptide of type I collagen; 25(OH)D: 25-hydroxyvitamin D. * $p < 0.05$, ** $p < 0.001$.

independent predictors of sRANKL (adjusted $R^2 = 0.19$, $p < 0.01$), the variables significantly associated with sRANKL were platelet aggregation inhibitor (ß = 0.28, p = 0.047), hip BMD (ß = –0.33, p = 0.02), and OC (ß = 0.33, p = 0.02).

DISCUSSION

The most original finding of our study is the increase of sRANKL levels and sRANKL/OPG ratio in patients with SSc. They did not correlate with sVCAM and did not show differential patterns as a function of clinical variables, apart from treatment with platelet aggregation inhibitors; at present we have no explanation for these findings. Interestingly, sRANKL and sRANKL/OPG ratio correlated negatively with BMD measures. We found distinctly lower 25(OH)D levels in patients versus controls; conceivably hypovitaminosis D (through malabsorption and decreased sunlight exposure) is pathogenetically important for the increased prevalence of osteoporosis in patients with SSc[33,34]. It is likely, therefore, that raised sRANKL levels reflect more an altered bone microenvironment than vascular damage. Previous studies looking at correlations between serum sRANKL levels and bone mass or bone turnover markers in healthy women have yielded conflicting results, with authors reporting negative[27,35-37], positive[38,39], or no association[40-42] with bone density. Reasons accounting for such discrepancies are not obvious.

We did not find differences between patients and controls for bone formation markers, while CTX levels were marginally higher in patients. While the observed parallel between CTX on the one hand and OC and bALP on the other confirms that this analyte reflects bone turnover, the negative correlation with 25(OH)D levels could suggest a condition of secondary hyperparathyroidism leading to increased bone turnover. Extraskeletal sources of CTX have been suggested by Allanore, *et al*[43], who reported increased levels in patients with SSc, and also correlations with cutaneous and pulmonary involvement, suggesting CTX as a reliable marker of disease activity. At variance with these findings, we did not note any correlation of CTX with disease variables.

In our study, OPG levels were not statistically different in patients and controls; in both groups they correlated positively with age, as reported[16]. Interestingly, in univariate analysis OPG levels correlated positively with estimates of cardiovascular and coronary risk in patients with SSc, similar to what we found in a study of patients with Cushing's syndrome[29]. At variance with OPG, the levels of sVCAM, an established marker of endothelial activation/injury, were consistently and markedly supranormal in our patients, in

accord with other reports[44,45]. In addition, sVCAM levels also correlated positively with estimates of cardiovascular and coronary risk. It is held that serum sVCAM and OPG are both increased in patients with atherosclerotic disease[16,46,47]; one could argue, therefore, that circulating sVCAM and OPG reflect an overall cardiovascular risk, and behave similarly in patients with atherosclerosis but diverge in patients with SSc. One possible explanation is that in such patients sVCAM reflects endothelial injury in both micro- and macrovascular disease[44-47], while OPG reflects only macrovascular disease, possibly as a consequence of the involvement or calcification of the arterial media[16,19]; moreover, OPG is somewhat influenced by disease variables. Higher levels in patients with calcinosis may be viewed as an inadequate compensatory response of OPG as an inhibitor of calcification[15], while association with active ulcers is difficult to explain. The negative correlation of OPG with CTX may reflect the role of OPG as an inhibitor of bone resorption, even if no correlation with BMD measures was found.

Limitations of our study require comment. The cross-sectional design cannot provide information about dynamics of the examined analytes as a function of disease progression and effect of therapy. Recruitment of patients with SSc at diagnosis would have avoided the interference of treatments and limited the heterogeneity of the cohort. Addressing this point, however, is difficult due to low incidence and diagnostic delay of the disease. Moreover, we did not assess direct indexes of endothelial dysfunction (such as flow-mediated brachial artery dilation) and macrovascular involvement (such as carotid intima-media thickness); estimates of cardiovascular and coronary risk are based on classical risk factors and may not take into account disease-specific mechanisms contributing to cardiovascular events[5]. In addition, we did not assess bone density in control subjects to evaluate possible excess prevalence of osteoporosis in patients with SSc[33,34]. Finally, due to the relatively small number of cases, subgroup analyses should be considered as exploratory and hypothesis-generating rather than conclusive.

We report higher sRANKL levels and sRANKL/OPG ratio in patients with SSc, seemingly as a consequence of altered bone microenvironment. We also report a dissociation between the established marker of endothelial activation/injury sVCAM and the alleged marker of vascular damage OPG in patients with SSc. Further studies are needed to clarify the relationships of the RANKL/RANK/OPG system with the progression of macro- and microvascular damage in SSc.

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