Clinical Cancer Research

Prognostic Value of RANKL/OPG Serum Levels and Disseminated Tumor Cells in Nonmetastatic Breast Cancer

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Abstract

Purpose: We assessed serum concentrations of the receptor activator of NFκB ligand (RANKL) and its decoy receptor, osteoprotegerin (OPG), two proteins implicated in the development and progression of breast cancer, in 509 patients with primary, nonmetastatic breast cancer. Then the results were evaluated with regards to the occurrence of bone metastases, the presence of disseminated tumor cells (DTC) in the bone marrow, survival, and risk of developing metastatic disease.

Experimental Design: Before surgery, two bone marrow aspirates were analyzed for DTC using density centrifugation followed by immunocytochemistry (pan-cytokeratin antibody A45-B/B3). RANKL and OPG levels in the serum were measured by ELISA.

Results: RANKL levels were significantly lower in women >60 years ($P < 0.0001$) and RANKL/OPG ratios higher in lymph node–positive patients ($P < 0.05$). High OPG serum levels were associated with a higher risk of death from breast cancer [HR 1.94; 95% confidence interval (CI) 1.23–3.07; $P = 0.005$] and OPG was an independent prognostic marker for breast cancer–specific survival (BCSS; multivariate analyses, $P = 0.035$). RANKL levels were 33% higher ($P < 0.0001$) in DTC^{pos} patients (41%), whereas high levels were associated with a significantly better BCSS in DTC^{neg} patients as compared with low levels (HR 0.524; 95% CI 0.30–0.95; $P = 0.04$). RANKL serum levels were significantly increased in patients who developed bone metastases ($P = 0.01$) and patients within the highest quartile of RANKL had a significantly increased risk of developing bone metastases compared with those in the lowest (HR 4.62; 95% CI 1.49–14.34; $P = 0.03$).

Conclusions: These findings warrant further investigation as they provide a rationale for novel diagnostic or therapeutic approaches.

Introduction

Despite major improvements in diagnosis and treatment, patients with breast cancer are prone to bone metastasis, which often occur many years after the initial diagnosis (1). This relapse may be explained by an early micrometastatic spread of disseminated tumor cells (DTC) to the bone marrow, which is detectable in up to 40% of the patients (2, 3). The malignant character of DTCs has already been demonstrated and the presence and persistence of these cells have been widely accepted as an independent prognostic marker of decreased progression-free survival (PFS) and overall survival (OS; refs. 4–9). As a limiting factor, the analysis of DTCs as a monitoring tool requires an invasive and painful procedure for the patients. For routine monitoring of disease progression and prognosis, more convenient and cost-effective serum or plasma-based tests are warranted to estimate the risk of bone metastases.

Bone metastases, secondary to breast cancer, generally have an osteolytic appearance and are susceptible to pathologic fracture. At the cellular level, osteoclastic bone resorption is enhanced, whereas bone formation is impaired, resulting in accelerated bone loss (10). Although the inhibition of osteoblast function by cancer-secreted proteins like the Wnt inhibitor Dickkopf-1 deserves consideration, an enhanced action of osteoclastic bone resorption is regarded as the hallmark of metastatic progression in the bone (11).

Osteoclasts are regulated by the receptor activator of NFκB ligand (RANKL). RANKL is secreted by osteoblasts and osteocytes and regulates the differentiation and activity of osteoclasts by binding to its receptor activator of NFκB (RANK), which is expressed on osteoclasts and osteoclast precursors (12).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Receptor activator of NFκB ligand (RANKL) and its decoy receptor osteoprotegerin (OPG) are important regulators of bone homeostasis that have been recently implicated in the development and progression of breast cancer. However, their biology appears increasingly complex. We demonstrate that high levels of OPG are an independent prognostic marker for breast cancer–specific survival (BCSS), whereas high RANKL levels indicate an improved BCSS in disseminated tumor cell (DTC)-negative patients. RANKL levels were increased in DTC-positive patients and in patients who later developed bone metastases. In light of the recent controversial results with regard to the clinical benefit of denosumab, an antibody targeting RANKL, in the prevention of bone metastases in patients with breast cancer, these findings warrant further investigations of RANKL and OPG as biomarkers and mediators of breast cancer progression and metastatic bone disease.

Osteoprotegerin (OPG) is a soluble decoy receptor that can bind and inhibit the interaction of RANKL with RANK. In addition to its effects on osteoclasts, RANKL has been shown to exert direct effects on malignant cells. RANKL is a mediator of progestin-driven breast carcinogenesis (13, 14) and also promotes the migration of highly osteolytic breast and melanoma cells in preclinical models of bone metastases (15). Several studies have shown that the expression of RANK on cancer cells is a negative prognostic marker and associated with an increased risk of bone metastases (16, 17). Despite these findings, little is known about the prognostic value of serum RANKL and OPG levels in breast cancer. This study aimed to assess RANKL and corresponding OPG levels in serum samples of women with primary, nonmetastatic breast cancer at the time of first diagnosis, and to investigate whether these findings are associated with the prognosis, clinical parameters, as well as the presence of DTCs in the bone marrow and the occurrence of bone metastases.

Materials and Methods

Patient population, patient characteristics, and study design

We retrospectively analyzed a cohort of 509 primary, patients with nonmetastatic breast cancer, diagnosed between 2004 and 2009, before the onset of therapy. All specimens were obtained and collected after written informed consent from all subjects using protocols approved by the clinical Ethic committee of the University Hospital Essen (05/2856) and conducted in accordance with the Declaration of Helsinki.

The eligibility criteria were: histologically proven breast cancer, bone marrow aspiration at the time of primary diagnosis, no severe uncontrolled comorbidities or medical conditions, no further present malignancies or malignancies in the past, completion of adjuvant treatment according to the current guidelines (18) including adjuvant chemotherapy (anthracyclines, 5-fluorouracil, taxanes, and cyclophosphamide), antihormonal therapy in case of hormone-responsive tumors (tamoxifen or an aromatase inhibitor), trastuzumab in the case of HER2-positivity (after FDA approval in November 2006), and radiotherapy if indicated (Supplementary Table S1). Patients treated with neoadjuvant chemotherapy were excluded. For each of the 509 patients, the tumor type, tumor–node–metastasis (TNM) staging, and grading were assessed at the Institute of Pathology of the University Hospital Essen (Essen, Germany) as part of the West German Comprehensive Cancer Center. Patients positive for DTCs in the bone marrow were recommended to complete a prescription of clodronate (2 × 520 mg/day) for at least two years. This recommendation was based on data from Diel and colleagues, which showed that clodronate prolonged OS as compared with an observation group and reduced the frequency of bone as well as visceral metastases (19).

Selection and detection of DTCs

Between 10 and 20 mL bone marrow was aspirated from the anterior iliac crests of all patients at the beginning of surgery of the primary tumor, before start of any therapy and processed within 24 hours. DTC isolation and detection was performed on the basis of the recommendations for standardized tumor cell detection, published by the German Consensus group of Senology (20). Details of the staining procedure, for example, number of evaluated slides, controls, and cell detection have been described elsewhere (21). Briefly, bone marrow cells were isolated from heparinized bone marrow (5,000 U/mL bone amrrow) by Ficoll–Hypaque density gradient centrifugation (density 1.077 g/mol; Pharmacia) at 400 × *g* for 30 minutes. Slides were analyzed for DTCs by immunocytochemistry using the pan-cytokeratin antibody A45-B/B3. Microscopic evaluation of the slides was carried out using the ARIOL system (Applied Imaging), according to the ISHAGE evaluation criteria (22).

Sampling of serum

Nine milliliters of blood were collected with an S-Monovette (Sarstedt AG & Co.) from each patient, stored at 4°C, and processed within 4 hours to avoid blood cell lysis. Blood fractionation was carried out by centrifugation for 10 minutes at 2,500 × *g*. Subsequently, 3–4 mL of the upper phase, constituting blood serum, were removed and stored at −80°C. All samples were assayed in batch form for RANKL and OPG.

Detection of RANKL and OPG by ELISA

RANKL serum levels were detected by ELISA (Biomedica). Briefly, 150 μL of undiluted serum was used and processed according to the protocol provided. Following sample incubation overnight, wells were washed and substrate was added as instructed. Absorbance was measured immediately at 450 nm with reference at 630 nm using FLUOstar Omega (BMG Labtech). Assay characteristics are as follows: lower detection limit is 0.01 pmol/L, intraassay precision ≤4%, and interassay precision ≤3%. The assay is specific for endogenous and recombinant human-free soluble RANKL.

OPG serum levels were detected by ELISA (Biomedica). Twenty microliters of serum were incubated with the biotinylated anti-OPG antibody for 4 hours, followed by the recommended washing steps. Subsequently, the conjugate buffer was added for another hour and after another set of washing steps, the substrate was added. After 30 minutes, the reaction was stopped and absorbance was measured immediately at 450 nm with reference at 630 nm. Assay characteristics are described as follows: intraassay precision ≤3%, interassay precision ≤5%. The assay is specific for human OPG.

Statistical analysis

Results are presented as SD of the mean, unless otherwise stated. Groups of two were assessed by the Mann–Whitney *U* test, groups of three or more were assessed by ANOVA. A *P* value of <0.05 was considered statistically significant.

Unless otherwise stated, serum samples were divided into two groups at the RANKL, OPG or RANKL-to-OPG ratio median and classified as high or low groups. The 10 samples that showed RANKL levels below the detection limit were allocated to the RANKL-low group. Kaplan–Meier curves were assessed using the log-rank (Mantel–Cox) test. Breast cancer–specific survival (BCSS) was defined as time between diagnosis of the primary tumour and death directly related to the disease. For OS, death of any cause was considered as endpoint. Multivariate Cox regression analyses were performed to identify prognostic factors for the different survival endpoints. The multivariate Cox regression models were adjusted to known clinical prognostic factors in patients with breast cancer. *P* values <0.05 were considered statistically significant.

Results

Cohort

The assessed cohort consisted of 509 patients. Their clinical characteristics at the time of initial diagnosis are shown in Table 1. With a median follow-up of 8.50 years (range 0.16–13.64), survival data were available for 504 patients (5 patients lost to follow-up). The median age of women included was 60 years (range 27 to 86 years) and the majority of the patients were postmenopausal (374/509, 73%). Most patients had T1 tumors, 324/509 (64%), 340/509 (67%) of the patients were lymph-node–negative and most patients had a moderately differentiated tumor (271/509, 53%). Expression of the estrogen (ER) and progesterone receptor (PR) was observed in 81% (414/509) and 73% (373/509) of the tumors, respectively. HER2 was overexpressed in 16% (80/509) of the tumors. Analyzing subgroups based on the hormone receptor status and HER2 expression, 72% (364/509) were ER- and/or PR- positive and HER2-negative, 13% (65/509) were triple-negative, 11% (57/509) were triple-positive, and 5% (23/509) only showed HER2 overexpression.

Table 1. Clinical data of patients

	Total (%)
Total	509
Age (years)	
<60	244 (48)
>60	265 (52)
Menopausal status	
Premenopausal	72 (14)
Perimenopausal	63 (12)
Postmenopausal	374 (73)
Histology	
Ductal	385 (77)
Lobular	68 (13)
Others	56 (11)
Tumor stage	
pT1	324 (64)
pT2	160 (31)
pT3–4	24 (5)
Unknown	1 (0.2)
Nodal status	
Node negative	340 (67)
Node positive	167 (33)
Unknown	2 (0.4)
Grading	
I	89 (17)
II	271 (53)
III	148 (29)
Unknown	1 (0.2)
Lymphangiosis	
Negative	407 (80)
Positive	98 (19)
Unknown	4 (1)
Hemangiosis	
Negative	495 (97)
Positive	7 (1)
Unknown	7 (1)
ER Status	
Negative	94 (18)
Positive	414 (81)
Unknown	2 (0.4)
PR Status	
Negative	135 (27)
Positive	373 (73)
Unknown	1 (0.2)
Her2 Status	
Negative	426 (84)
Positive	80 (16)
Unknown	3 (0.6)
IHC Subtype	
(ER^-, PR^-, $Her2^-$)	65 (13)
(ER^-, PR^-, $Her2^+$)	23 (5)
(ER^+ and/or PR^+, $Her2^-$)	364 (72)
(ER^+ and/or PR^+, $Her2^+$)	57 (11)
Bone marrow status (DTC status)	
Negative	300 (59)
Positive	207 (41)

NOTE: Patient characteristics are displayed as total number (*n*) and percentage of all (%). In case of DTC assessment, percentage of positive and negative is given from those assessed.

RANKL and OPG expression in patients with breast cancer at baseline

RANKL and OPG serum measurement was performed for all patients. Measurable levels of RANKL and OPG were detectable in 98% (499/509) and 100% (509/509) of samples, respectively. Of note, RANKL levels were below the detection limit in 10 cases. The mean serum value was 0.23 ± 0.20 pmol/L (range 0.001–1.36) for RANKL and 4.24 ± 1.68 pmol/L (range 0.46–13.40) for OPG. RANKL, OPG, and RANKL/OPG ratios were stratified according to age (<60 vs. >60 years), histology, TNM classification, menopausal status, and hormone receptor status (Table 2). In women below 60 years of age, RANKL levels were significantly higher than in older women (0.26 ± 0.22 pmol/L vs. 0.20 ± 0.15 pmol/L; $P < 0.0001$). OPG levels were lower in younger patients, resulting in a higher RANKL/OPG ratio in patients below 60 years of age (0.09 ± 0.10 vs. 0.05 ± 0.05; $P < 0.0001$). This finding was reflected when patients were stratified according to menopausal status. Premenopausal patients with a mean age of 42.6 years had higher RANKL (0.26 ± 0.22 pmol/L vs. 0.21 ± 0.17 pmol/L) and RANKL/OPG ratios (0.10 ± 0.11 vs. 0.06 ± 0.07) than postmenopausal patients (mean age 64.3 years). With regards to histology, no significant differences were observed for RANKL, OPG, and the RANKL-to-OPG ratio between groups. Both, RANKL and OPG levels were also unaltered between different tumor stages (T1–T4). However, RANKL levels (0.25 pmol/L ± 0.22 vs. 0.22 ± 0.19 pmol/L) and the

Table 2. RANKL, OPG, and RANKL/OPG ratios in patients with breast cancer

	RANKL (pmol/L)	*P*	OPG (pmol/L)	*P*	RANKL/OPG	*P*
Age (years)						
<60	0.26 ± 0.22	<0.0001	3.83 ± 1.44	<0.0001	0.09 ± 0.10	<0.0001
>60	0.20 ± 0.15		4.63 ± 1.80		0.05 ± 0.05	
Menopausal status						
Premenopausal	0.26 ± 0.22	<0.05 (vs. peri)	3.56 ± 1.66	<0.05 (vs. peri)	0.10 ± 0.11	<0.001 (vs. peri)
Perimenopausal	0.28 ± 0.26		3.80 ± 1.25	<0.01 (vs. pre)	0.09 ± 0.09	
Postmenopausal	0.21 ± 0.17		4.45 ± 1.71		0.06 ± 0.07	
Histology						
Ductal	0.24 ± 0.20	ns	4.20 ± 1.64	ns	0.07 ± 0.09	ns
Lobular	0.21 ± 0.16		4.51 ± 2.19		0.06 ± 0.06	
Others	0.20 ± 0.20		4.24 ± 1.23		0.05 ± 0.05	
Tumor stage						
pT1	0.22 ± 0.19	ns	4.24 ± 1.69	ns	0.07 ± 0.08	ns
pT2	0.23 ± 0.20		4.25 ± 1.75		0.07 ± 0.09	
pT3-4	0.21 ± 0.18		4.33 ± 1.26		0.07 ± 0.05	
Nodal status						
Node negative	0.22 ± 0.17	ns	4.25 ± 1.61	ns	0.07 ± 0.07	<0.05
Node positive	0.25 ± 0.22		4.22 ± 1.83		0.08 ± 0.10	
Grading						
I	0.22 ± 0.19	ns	4.21 ± 1.34	ns	0.06 ± 0.08	ns
II	0.22 ± 0.19		4.26 ± 1.84		0.07 ± 0.09	
III	0.24 ± 0.19		4.22 ± 1.59		0.07 ± 0.07	
ER Status						
Negative	0.20 ± 0.17	ns	4.10 ± 1.63	ns	0.06 ± 0.06	ns
Positive	0.23 ± 0.20		4.28 ± 1.70		0.07 ± 0.08	
PR Status						
Negative	0.20 ± 0.18	ns	4.21 ± 1.67	ns	0.06 ± 0.07	ns
Positive	0.24 ± 0.20		4.26 ± 1.69		0.07 ± 0.08	
Her2 Status						
Negative	0.23 ± 0.2	ns	4.25 ± 1.7	ns	0.07 ± 0.08	ns
Positive	0.20 ± 0.16		4.22 ± 1.62		0.06 ± 0.07	
Breast cancer subtypes						
ER^-, PR^-, $Her2^-$	0.22 ± 0.17	ns	4.27 ± 1.80	ns	0.06 ± 0.06	ns
ER^-, PR^-, $Her2^+$	0.18 ± 0.12		3.67 ± 1.21		0.06 ± 0.06	
ER^+ and/or PR^+, $Her2^-$	0.24 ± 0.20		4.24 ± 1.69		0.07 ± 0.08	
ER^+ and/or PR^+, $Her2^+$	0.21 ± 0.18		4.45 ± 1.59		0.06 ± 0.08	

NOTE: Values of RANKL and OPG are given in pmol/L. RANKL/OPG is displayed as a ratio of the two values. Endpoint age was separated at 60 years, as defined at the time of data analyses.
Abbreviation: ns, not significant

RANKL/OPG ratio were higher in patients with lymph node involvement (0.08 ± 0.07 vs. 0.07 ± 0.10; $P < 0.05$). There were no differences in RANKL levels and the RANKL/OPG ratio when patients were stratified into different breast cancer subtypes (bottom of Table 2).

Prognostic value of RANKL and OPG

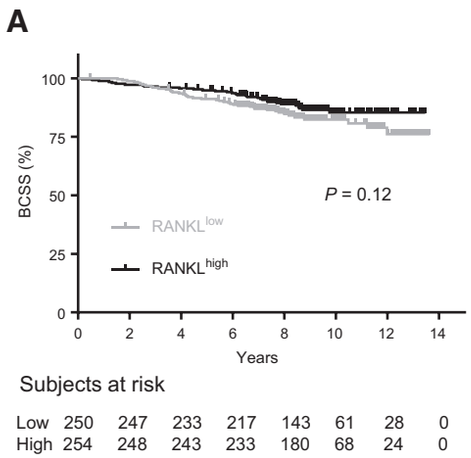
During the period of follow-up (mean 8.50 years, range 0.16–13.64), a total of 76 (15.1%) deaths were documented, with 74 (14.7%) of those being attributed to breast cancer. High serum levels of RANKL did not significantly affect breast cancer survival compared with low serum levels of RANKL [32/254 vs. 42/255; HR 0.70; 95% confidence interval (CI) 0.44–1.10; $P = 0.12$; Fig. 1A]. High levels of OPG, on the other hand, resulted in a significantly lower survival, compared with low levels of OPG (81.5% vs. 89.5%) with 47/254 and 27/255 reported cases of breast cancer–specific death (HR 1.94; 95% CI 1.23–3.07; $P = 0.005$), respectively (Fig. 1B). The RANKL/OPG ratio did not affect the survival of this cohort (Fig. 1C). When assessing survival differences between different subgroups, the fraction with $RANKL^{low}/OPG^{high}$ serum levels had the highest rate of breast cancer–specific deaths (30/125; 24%), whereas the lowest rate of death was seen in the $RANKL^{high}/OPG^{low}$ fraction (15/142; 10.6%; Supplementary Fig. S1). Multivariate analyses revealed OPG as an independent prognostic marker for BCSS ($P = 0.035$; Fig. 1D).

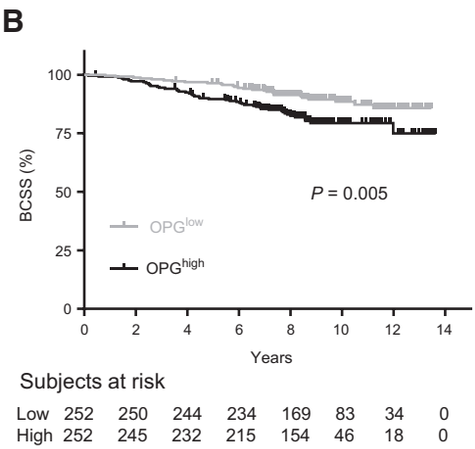
RANKL and OPG expression in patients with DTCs

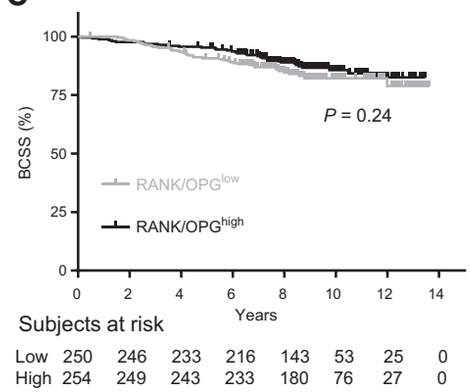
Bone marrow was studied in all patients, with 207/507 (41%) patients identified as DTC^{pos} (Table 1). As shown in Fig. 2, patients with DTCs in the bone marrow had significantly higher RANKL serum levels (+33%) than the group without DTCs (0.20 ± 0.16 vs. 0.27 ± 0.23; $P < 0.0001$). OPG levels did not differ between both groups. Elevated RANKL levels and unchanged OPG levels resulted in an elevated RANKL/OPG ratio in patients with bone marrow infiltration of DTCs (0.06 ± 0.07 vs. 0.09 ± 0.10; $P < 0.0001$).

Influence of DTC status on the prognostic value of RANKL and OPG

The DTC status had pronounced effects on the prognostic value of RANKL and OPG (Fig. 3). In DTC^{neg} patients, high serum levels of RANKL were associated with a significantly better BCSS compared with low levels (HR 0.524; 95% CI 0.30–0.95; $P = 0.04$). In DTC^{pos} patients, the serum levels of RANKL did not influence patient survival (HR 0.98; 95% CI 0.48–2.03; $P = 0.97$; Fig. 3). DTC^{neg} patients with high







D

	Univariate analyses			Multivariate analyses		
	HR	95% CI	*P*	HR	95% CI	*P*
RANKL	0.69	0.44–1.10	0.122	Not performed		
OPG	1.94	1.23–3.07	0.005	1.70	1.04–2.80	0.035

Adjusted for age, tumor stage (T), lymph node involvement (N), grade (G) and the expression of estrogen, progesterone receptor and HER2

Figure 1.
Prognostic value of RANKL, OPG, and RANKL/OPG. BCSS is significantly increased in patients with low OPG levels (**B**). RANKL and RANKL/OPG ratios do not impact BCSS (**A** and **C**). Multivariate analyses confirm the independent prognostic value of OPG (**D**).

levels of OPG had an increased risk to die from breast cancer than those with low levels (HR 1.91; 95% CI 1.08–3.51; $P = 0.03$), whereas only a trend was observed in the DTC^{pos} group (HR 1.96; 95% CI 0.95–4.01; $P = 0.07$). The RANKL/OPG ratio did not affect the prognosis of patients within the DTC^{pos} or DTC^{neg} group (data not shown). Multivariate analyses confirmed the prognostic significance of RANKL in the DTC^{neg} cohort, whereas OPG did not remain significant after multivariate adjustment in the DTC^{neg} group (Supplementary Fig. S2).

Occurrence of distant metastases

During the time of follow-up, distant metastases were detected in 30 of 413 evaluable patients (Table 3). No data were available for 96 patients. Median time to diagnosis of metastases was 4.00 years (range 0.014–11.32). In patients

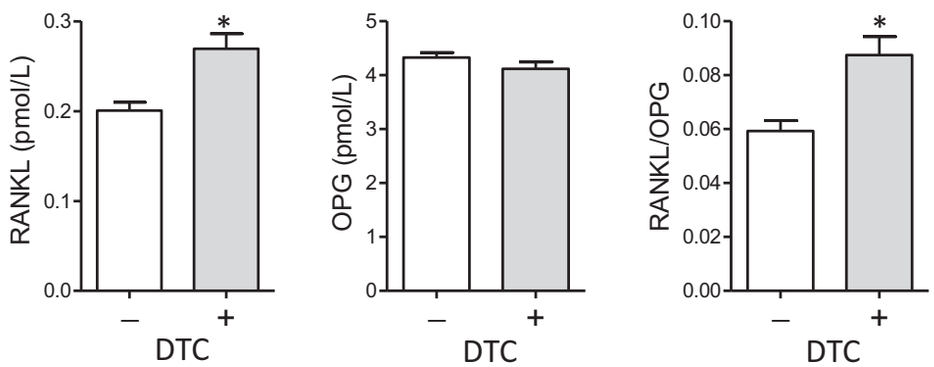


Figure 2.
RANKL and RANKL/OPG ratios are elevated in patients with DTC^{pos} breast cancer. Values of RANKL and OPG are given in pmol/L. RANKL/OPG is displayed as a ratio of the two values. Values are shown as mean ± SEM (*, $P < 0.0001$).

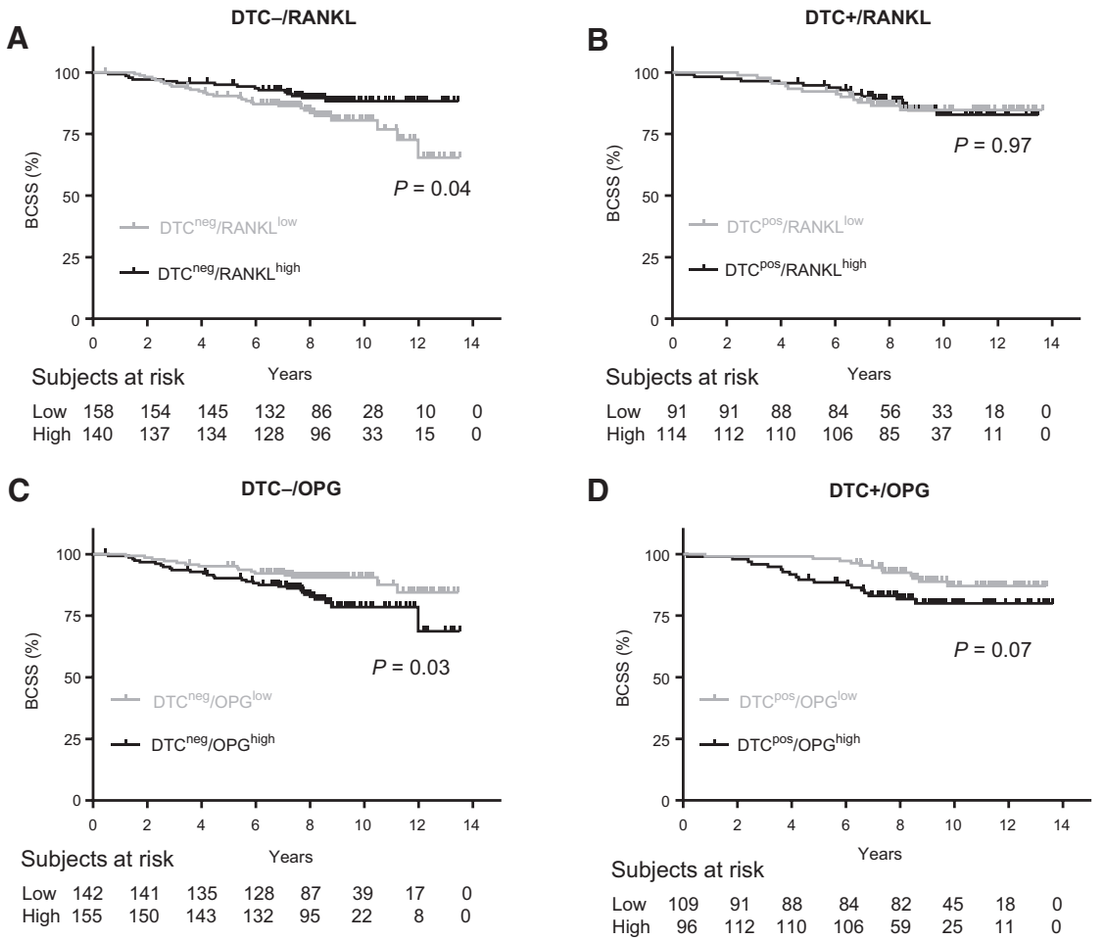


Figure 3.

RANKL and OPG are prognostic markers in DTC^{neg} breast cancer. Following separation into DTC^{neg} (**A** and **C**) and DTC^{pos} (**B** and **D**) groups, high levels of RANKL (**A**) and low levels of OPG (**C**) are associated with significantly improved prognosis in DTC^{neg} patients.

who developed metastases, RANKL ($P = 0.06$) and OPG ($P = 0.91$) levels were not significantly different when compared with controls. However, the RANKL/OPG ratio was higher in patients who developed distant metastases ($P = 0.006$). Assessment of the occurrence of metastases according to RANKL status revealed a numerical imbalance between patients with high and low RANKL levels. In the high RANKL cohort, the risk of developing distant metastases was increased by 72.7% compared with the RANKL low group (19 vs. 11 events). In the OPG-high and -low group, 17 and 13 cases of distant metastases were recorded, respectively. A high RANKL/OPG ratio was also associated with an increased occurrence of distant metastases (19 vs. 11 events).

Occurrence of bone metastases

Of the 30 patients with documented metastases, 23 were diagnosed with bone metastases. RANKL serum levels were significantly increased in patients that developed bone metastases compared with those that did not develop metastases ($P = 0.01$). No significant changes of OPG levels were found, but RANKL/OPG levels were significantly higher in patients that developed bone metastases ($P = 0.0004$; Table 3). The risk of developing

Table 3. RANKL, OPG, and RANKL/OPG ratios and the occurrence of distant metastases

	N (%)	RANKL	*P*	OPG	*P*	RANKL/OPG	*P*
Distant metastases (any site including bone)							
Positive	30/413 (7.3%)	0.30 ± 0.24		4.20 ± 2.17		0.11 ± 0.14	
Negative	383/413 (92.7%)	0.22 ± 0.19	0.056	4.22 ± 1.63	0.908	0.07 ± 0.08	**0.006**
Bone metastases							
Positive	23/413 (5.6%)	0.33 ± 0.25		4.25 ± 1.66		0.13 ± 0.15	
Negative	390/413 (94.4%)	0.23 ± 0.19	**0.01**	3.93 ± 2.00	0.38	0.07 ± 0.08	**0.0004**

NOTE: Values of RANKL and OPG are given in pmol/L. RANKL/OPG is displayed as a ratio of the two values. Significant values ($P < 0.05$) are shown in bold.

bone metastases was increased by 87.5% in the RANKL^{high} group ($n = 15$) compared with the RANKL^{low} group ($n = 8$). Levels of OPG had no effect on the incidence of bone metastases. When separating the group according to their RANKL/OPG ratio, 16 (7.88%) events of bone metastases occurred in the group with high RANKL/OPG levels, compared with 7 (3.45%) cases in the group with low RANKL/OPG levels ($P = 0.088$). Differences were even more apparent when comparing patients (with available follow up for distant relapse) in the lowest and highest quartile of RANKL serum levels ($n = 100$ each). There was a 5-fold increase in the risk of developing bone metastases in the highest RANKL quartile (10/100) compared with the lowest quartile (2/100; HR 4.62; 95% CI 1.49–14.34; $P = 0.03$; Fig. 4).

Discussion

The occurrence of bone metastases secondary to breast cancer, years or even decades after the initial diagnosis, is often explained by micrometastatic spread of DTCs to the bone marrow. The presence and persistence of DTCs is widely accepted as an independent prognostic marker (4–8) and the bone marrow is considered as a metastatic niche for DTCs from solid tumors and a potential reservoir for relapse (23, 24). Limiting its application, assessment of DTC status requires invasive bone marrow aspiration and more convenient ways to predict outcome are warranted. In recent years, circulating tumor cells (CTC) in peripheral blood have been increasingly investigated as a less invasive option and are now commonly used (25, 26). CTCs have been established as prognostic markers of disease recurrence, but high costs and time consumption remain limitations (27).

The RANKL/OPG system plays an important role in the pathogenesis of bone metastases. However, the biology of RANKL and OPG is complex and although often considered as one system, RANKL and OPG may exert independent effects on cancer cells. In this study, levels of RANKL did not statistically affect BCSS. High levels of OPG, on the other hand, were associated with a poorer survival. The highest risk of breast cancer–specific death was seen in the group with low RANKL and high OPG serum levels. Interestingly, we did not observe differences between groups when looking at the RANKL/OPG ratio, suggesting that effects may be mediated independently and our results emphasize the necessity of looking at both proteins individually.

OPG is best known for its role as a decoy receptor for RANKL (28). In this context high levels of OPG are considered as bone protective and the application of OPG can restore tumor induced bone loss in murine models of breast cancer bone metastases (29). However, OPG can also act as an inhibitor of TRAIL, which induces tumor cell apoptosis (30) and high levels of OPG were associated with a higher cancer-related mortality in a large observational study with more than 6,000 patients (31). High levels of OPG have conferred a poor prognosis in

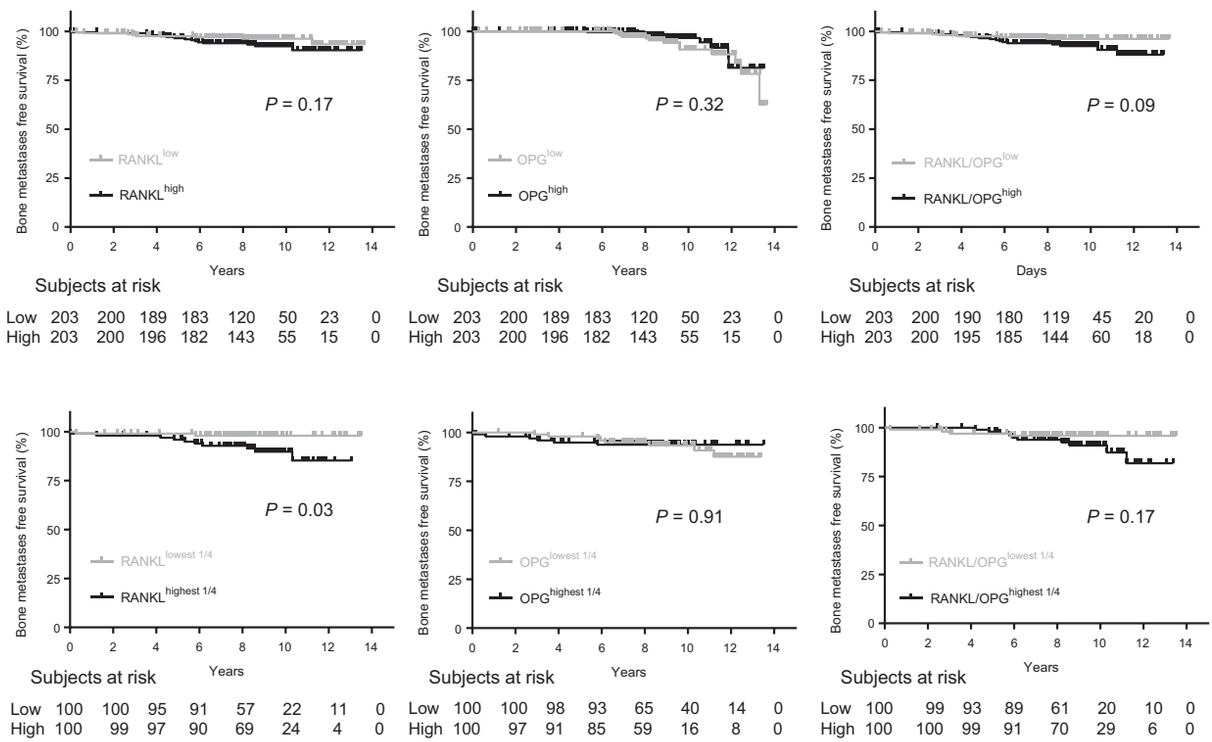


Figure 4.

Risk of bone metastases is increased in patients with the highest RANKL serum levels compared with the lowest RANKL levels. Bone metastases occur significantly more often in patients with the highest RANKL levels compared with the lowest RANKL levels. No differences are seen with OPG or RANKL/OPG ratios.

patients with metastatic colorectal cancer (32). In prostate cancer, increased levels of OPG have been observed in patients with advanced cancer (33, 34). In breast cancer, higher concentrations of OPG were associated with an increased risk of ER-negative breast cancer (35). In addition, preclinical studies indicate that OPG mediates tumor-promoting effects as a mediator of inflammation in breast cancer (36) and promotes metastases in triple-negative breast cancer (37). Our data are in line with these results, implying that high levels of OPG, although potentially bone protective, actually confer a poor prognosis in certain malignancies including breast cancer.

When dissecting patients according to their DTC status, neither RANKL nor OPG affected the prognosis in DTC^{pos} patients, but DTC^{neg} patients had a significantly worse overall prognosis with low RANKL ($P = 0.04$) or high OPG ($P = 0.01$) levels. Interestingly, after applying correction for multiple prognostic markers, the prognostic significance of RANKL remained intact but was lost for OPG (Supplementary Fig. S1). These results suggest that RANKL may exert effects dependent on the DTC status of the patients. The role of RANKL in breast cancer appears especially complex and previous studies have described RANKL both as a positive and negative prognostic factor. In the past years, a role of RANKL in the pathogenesis of progestin-driven mammary carcinoma has been suggested (13, 14). In postmenopausal women, high RANKL and progesterone serum levels stratify a subpopulation of women that are at high risk of developing breast cancer and RANKL/OPG ratios change depending on the presence of CTCs in patients with established breast cancer (38). A recent paper showed that low RANKL mRNA in early breast cancer tissue is associated with an increased risk of relapse and metastases (39). In another study, patients with low RANKL expression were more likely to develop local recurrence or die from the disease (40). In a different paper, dual expression of RANK and RANKL conferred a negative prognosis and RANKL appeared as an independent prognostic factor (41).

Some of these results appear counterintuitive at first, given that RANKL plays an important role in the pathogenesis of bone metastases and the presence of its receptor RANK on breast cancer cells has been previously associated with an increased occurrence of bone metastases and a poorer prognosis (18). However, different reports have also suggested that low levels of RANK and RANKL expression in the breast cancer tissue confer a poorer prognosis (42). In fact, the interaction of the RANKL/RANK pathway with regards to the bone-related outcome of patients breast cancer appears complex, and certain nucleotide polymorphisms in RANK and RANKL genes have been associated with a poorer bone metastasis-free survival (43).

In a recent publication, the expression of RANK-c, which is a RANK isoform produced through alternative splicing, has been shown to attenuate the aggressive properties of ER-negative breast cancer (44). Notably, the majority of studies have investigated the tissue expression of RANKL in the tumor. These results may vary significantly from RANKL levels in the circulation and serum and tissue analyses should be viewed separately. We here documented that low levels of RANKL were associated with poorer survival in DTC^{neg} patients, but RANKL serum levels and RANKL/OPG ratios were increased in patients with detectable DTCs in the bone marrow, prior to the establishment of detectable bone metastases, indicating subtle activation of the bone microenvironment. Furthermore, patients within the highest RANKL quartile had a significantly increased risk of developing bone metastases, compared with the lowest quartile and RANKL serum levels were significantly higher in patients that later developed bone metastases, compared with those that did not.

Our paper has certain strengths and limitations. Strengths include the large sample size and excellent characterization of the cohort with DTC assessment as well as the long and detailed follow-up. Limitations include the absence of an age-matched control group, which would have given further insights, especially with regard to changes in RANKL and OPG in normal healthy aging. Furthermore, sequential RANKL and OPG measurements in the study cohort would have been desirable, as this may have given information on how changes in RANKL and OPG could influence prognosis. However, due to the fact that patients during the respective time frame were mostly monitored outside our clinic, these requirements were difficult to fulfill. Both RANKL and OPG show quite a big range, which may ultimately limit their ability as standard laboratory markers. As mentioned before, bone metastases may occur decades after initial diagnosis. With a median follow-up of 8.5 years, we may have missed some later occurring metastases and we cannot exclude that these may have affected our results.

Of note, per protocol, all patients with detectable DTCs were offered adjuvant bisphosphonate (clodronate intake for the duration of at least two years) and the majority of the patients (86%) followed that recommendation, including 7% of patients receiving zoledronic acid. We have recently published that the intake of bisphosphonates reduced the risk of DTC^{pos} patients to levels comparable with the results obtained for DTC^{neg} patients (20). Four other small pilot studies have described that both clodronate as well as zoledronic acid contributed to the eradication of DTCs, even years after the initial diagnosis (45–48). As a limitation, we cannot exclude that the concurrent use of bisphosphonates influenced the prognostic value of RANKL in the DTC^{pos} group.

The importance of RANKL in bone biology has resulted in the development and approval of denosumab, a mAb that inhibits RANKL, for the treatment of osteoporosis and bone metastases (49). For patients with prostate cancer, it has been demonstrated that denosumab delays the occurrence of bone metastases by a median of 4.2 months compared with the placebo group (50). In patients breast cancer, the ABCSG-18 trial investigated the effects of adjuvant denosumab on fracture reduction in postmenopausal women with breast cancer receiving aromatase inhibitors. In this trial, denosumab reduced the fracture risk independent of the patients T-score (51) and a significantly improved disease-free survival was found (52). Most recently, results from the D-CARE study (NCT01077154) were presented which aimed to establish the ability of denosumab to prevent the occurrence of bone metastases in patients with breast cancer with a high risk of developing metastatic bone disease. In this trial, RANKL inhibition with denosumab failed to reduce the rate of bone metastases (53).

These findings highlight the complex role of RANKL in breast cancer. Our results support the notion that RANKL and OPG may exert very specific, individual, and context-dependent effects on bone metastases and breast cancer biology and further studies are warranted to define specific breast cancer populations that may profit from RANKL inhibition. Furthermore, more attention should be given to the potential of OPG as an independent prognostic marker in breast cancer.

Disclosure of Potential Conflicts of Interest

T.D. Rachner reports receiving speakers bureau honoraria from Amgen. S. Kasimi-Bauer is a consultant/advisory board member for Qiagen. M. Rauner reports receiving speakers bureau honoraria from Amgen. L.C. Hofbauer is a consultant/advisory board member for Amgen. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: O. Hoffmann, R. Kimmig

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