Research Article

Cancer Prevention Research

Circulating RANKL and RANKL/OPG and Breast Cancer Risk by ER and PR Subtype: Results from the EPIC Cohort

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Abstract

Receptor activator of nuclear factor-kappa B (RANK)-RANK ligand (RANKL) signaling promotes mammary tumor development in experimental models. Circulating concentrations of soluble RANKL (sRANKL) may influence breast cancer risk via activation of RANK signaling; this may be modulated by osteoprotegerin (OPG), the decoy receptor for RANKL. sRANKL and breast cancer risk by hormone receptor subtype has not previously been investigated. A case–control study was nested in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. This study included 1,976 incident invasive breast cancer cases [estrogen receptor positive (ER+), $n = 1{,}598$], matched 1:1 to controls. Women were pre- or postmenopausal at blood collection. Serum sRANKL was quantified using an ELISA, serum OPG using an electrochemiluminescent assay. Risk ratios (RR) and 95% confidence intervals (95% CI) were calculated using conditional logistic regression. Associations between sRANKL and breast cancer risk differed by tumor hormone receptor status ($P_{het} = 0.05$). Higher concentrations of sRANKL were positively associated with risk of ER+ breast cancer [5th vs. 1st quintile RR 1.28 (95% CI, 1.01–1.63); $P_{trend} = 0.20$], but not ER− disease. For both ER+ and estrogen and progesterone receptor positive (ER+PR+) breast cancer, results considering the sRANKL/OPG ratio were similar to those for sRANKL; we observed a suggestive inverse association between the ratio and ER−PR− disease [5th vs. 1st quintile RR = 0.60 (0.31–1.14); $P_{trend} = 0.03$]. This study provides the first large-scale prospective data on circulating sRANKL and breast cancer. We observed limited evidence for an association between sRANKL and breast cancer risk. *Cancer Prev Res; 10(9); 525–34. ©2017 AACR.*

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

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doi: 10.1158/1940-6207.CAPR-17-0125

Introduction

The receptor activator of nuclear factor kappa-B (RANK) axis includes three tumor necrosis superfamily members; a transmembrane receptor (RANK), its only known ligand (RANKL), and a decoy receptor for RANKL (osteoprotegerin, OPG). The RANK-axis was first described in relation to its role in bone metabolism; the interplay between RANK, RANKL, and OPG regulates osteoclast development and activation, and is essential in bone homeostasis (1).

RANK and OPG protein and mRNA are expressed in multiple tissues and organs such as the adrenal gland, small intestine, thymus, and the breast (2–4). RANKL protein and mRNA is highly expressed in lung and lymph nodes and is found at lower levels in numerous other tissues including skeletal muscle, placenta, and heart (2). OPG and RANKL (in its soluble form, sRANKL) are also found in circulation (3–5).

RANKL expression in mammary epithelial cells is upregulated in pregnancy, and is essential for development of the lobuloalveolar mammary structures and the formation of a lactating mammary gland (3, 6, 7). In experimental models, the synthetic progesterone analogue medroxyprogesterone acetate (MPA) induces RANKL protein and mRNA expression in PR+ luminal mammary epithelial cells, resulting in auto-/paracrine stimulation of RANKL signaling in the mammary epithelium (8, 9). This triggers proliferation of mammary epithelial cells, expansion of mammary stem cells, and shields these cells from apoptosis, which results in increased rates of tumor formation (8, 9). In the human breast, expression of RANKL protein or mRNA is regulated by sex hormones and may be induced by progesterone and prolactin (3, 10, 11). RANKL expression in the human breast is correlated with high serum progesterone levels, and is required for progesterone-induced proliferation (10).

Human epidemiologic data on the RANK-axis and breast cancer risk is limited. Four studies to date have investigated circulating OPG (12–15) and breast cancer risk, one of which in a population of high-risk women (14). Only one study, conducted by our group, has investigated OPG and breast cancer risk by hormone receptor subtype (12). We observed a significant positive association between OPG concentrations and estrogen receptor (ER) negative disease in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, yet only a suggestive inverse association for ER positive cancers [third vs. first tertile RR: ER− 1.93 (95% CI, 1.24–3.02); $P_{trend} = 0.03$ and ER+ 0.84 (95% CI, 0.68–1.04); $P_{trend} = 0.18$. $n = 2{,}008$ total breast cancer cases; ref. 12]. Vik and colleagues observed a significant inverse association between OPG and breast cancer risk overall among 76 breast cancer cases (13) as did Odén and colleagues, in a small cohort of BRCA1 and BRCA2 carriers (18 breast cancer cases; ref. 14). In the only study to date to evaluate sRANKL and breast cancer risk, Kiechl and colleagues reported a positive association between sRANKL and breast cancer risk in postmenopausal women with relatively high circulating progesterone concentrations diagnosed 12 to 24 months after blood collection ($n = 21$ cases in this subgroup; ref. 15).

The RANK-axis has gained interest in breast cancer research as denosumab, a monoclonal antibody that inhibits RANKL, has been shown to reduce the number of skeletal related events (e.g., pathologic fracture) in cancer patients with bone metastasis (16) and has been suggested as a candidate for breast cancer prevention in women at high risk (17). Following our investigation of OPG in breast cancer (12), we conducted the first large-scale investigation on sRANKL and the sRANKL/OPG ratio and breast cancer risk in a nested case-control study in the EPIC cohort.

Materials and Methods

The EPIC started in 1992 to 2000 and follows 520,000 healthy adults (367,993 women) ages 35 to 75 years from 23 centers in 10 European countries (18). Incident cancer cases were identified through cancer registries in Denmark, Italy (except Naples), the Netherlands, Norway, Sweden, Spain, and the United Kingdom, and through review of health insurance records, contact with cancer and pathology registries, and/or direct contact with cohort members in France, Germany, Greece, and the Naples, Italy center. Mortality data were obtained via active follow-up in Germany and Greece, and via national and regional mortality registries in the remaining countries.

Detailed dietary, reproductive, lifestyle, anthropometric, and medical history data were collected through standardized methods. The majority of women (64%; $n = 235{,}607$) gave a blood sample. Blood samples were collected according to standardized protocols. For all countries, except Denmark and Sweden, half of the aliquots were stored locally and the other half centrally at the International Agency for Research on Cancer (IARC). The samples used in this study were stored at IARC are stored under liquid nitrogen at −196°C, or locally at −150°C for Danish participants. Participants from Sweden were not included in this study, as independent studies on breast cancer risk were conducted in those centers.

The study protocol for this study was approved by the ethical committees of the IARC (Project No. 12-42) and the University of Heidelberg (Project No. S311/2014). The EPIC study protocol was approved by the ethical committees of IARC and the participating centers. All participants gave written informed consent.

Nested case–control study

This study used a case–control design nested within the EPIC cohort. The study design and methods have been described previously (12, 19, 20). Briefly, breast cancer cases included in this case–control study were female and were diagnosed with a first invasive breast cancer between blood collection and completion of last follow-up, which ranged from 2003 to 2006 between centers. Both pre- and postmenopausal women were included; premenopausal women were all nonusers of oral contraceptives/exogenous hormones at blood collection, whereas postmenopausal women include both postmenopausal hormone (PMH) users and nonusers. Prior to 2004, all cases with available ER status were included. From 2004, among postmenopausal women, all incident ER− cases were included along with one ER+ case for every ER− case (matched on center). This investigation is limited to cases with ER status available; progesterone receptor (PR) status was available for 74% of cases. Controls were randomly selected from cohort participants who donated a blood sample and were alive and cancer free (except nonmelanoma skin cancer) at the time of diagnosis of the index case. Controls were matched on recruitment center, age (±3 months), menopausal status, PMH use, fasting status (<3, 3–6, and >6 hours), and time of the day (±1 hour) at blood donation. Premenopausal cases and controls were also matched on menstrual cycle phase at blood donation (early follicular, late follicular; peri-ovulatory, early luteal, mid luteal, late luteal). A total of 2020 case–control sets were selected for this study.

Laboratory analyses

Concentrations of sRANKL and OPG were analyzed at the Laboratory of the Division of Cancer Epidemiology at the German Cancer Research Center (DKFZ). Serum OPG was quantified using an electrochemiluminescence assay (MesoScale Diagnostics). Serum sRANKL was analyzed in duplicate, using an ELISA (Biomedica). Samples from cases and their matched controls were analyzed in the same analytical batch and laboratory personnel were blinded to the case–control status of the samples. The precision of the laboratory work was monitored by inclusion of blinded pooled quality control samples (2 per batch). Inter-batch coefficients of variation were 0.9% for premenopausal and 1.5% in postmenopausal women for sRANKL and 16.4% and 16.8%, respectively, for OPG. Intra-batch coefficients of variation for sRANKL were 15.6% for premenopausal and 13.3% for postmenopausal women. For OPG, these were 9.0% and 21.7%, respectively.

Assays for estradiol, estrone, testosterone, sex hormone-binding globulin (SHBG), insulin-like growth factor I (IGF-I), prolactin, progesterone, vitamin D and C-peptide in subsets of participants ($n = 611$ to 2,020) in this study were previously conducted at the IARC and the DKFZ (19–24).

Of the 2,020 case–control sets initially selected for this study, sRANKL concentrations were not available for 44 total case–control sets (38 sets, equipment failure and insufficient sample volume to re-assay; six sets, missing values). A total of 1,976 case–control sets remained for sRANKL analyses. For analyses including OPG, an additional nine case–control sets were excluded (four sets, missing values; five sets excluded due to outlying OPG values); 1,967 case–control sets were included for sRANKL/OPG ratio analyses. A total of 327 observations (8.1%; 175 cases, 152 controls) were below the limit of detection for sRANKL. These were set to 50% of the lower limit of detection of the assay (LLOD; 0.01 pmol/L).

Statistical analyses

Concentrations of sRANKL and OPG (both in in pmol/L), as well as the other available biomarkers, were $\log_2$ transformed to obtain approximately normal distributions. This transformation also allowed evaluation of the effect of a doubling in biomarker concentrations. The extreme studentized deviate test was used to evaluate outliers (25). The ratio was calculated as sRANKL concentration divided by OPG concentration; the ratio was then $\log_2$ transformed.

Cross-sectional correlations between sRANKL and endogenous hormones by menopausal status and postmenopausal hormone use at blood collection were assessed among study controls using partial Spearman correlations, adjusting for matching factors. sRANKL and the sRANKL/OPG ratio were classified into quintiles based on their distribution in controls. Conditional logistic regression was used to estimate risk ratios (RR) and 95% confidence intervals (CI) for breast cancer risk. Tests for trend were conducted using the continuous ($\log_2$) variables.

Multivariable models were adjusted for BMI (continuous; allowing separate associations by menopausal status), number of full-term pregnancies (0, 1, 2, ≥3, missing), and ages at menarche (≤12, 13, 14, ≥15, missing), first full-term pregnancy (no full-term pregnancy, ≤25, 25–29, ≥30, missing), and menopause (≤43, 44–47, 48–50, 51–52, 53–54, ≥55, missing). Additional adjustment for lifestyle and reproductive factors (e.g., smoking status, alcohol consumption, physical activity, use of exogenous hormones, breastfeeding) and endogenous hormones did not change the effect estimate by a factor of 1.10 (or the reciprocal). In addition to evaluating the sRANKL/OPG ratio, we evaluated the association between sRANKL and breast cancer, adjusted for OPG as a covariate in the logistic regression models, and evaluated the joint distribution of sRANKL and OPG by cross-classifying both markers at the median value (e.g., comparing sRANKL >median/OPG <median to sRANKL <median/OPG >median).

We assessed heterogeneity by reproductive and lifestyle factors (e.g., menopausal status, use of exogenous hormones, number of full term pregnancies, smoking status) and endogenous hormones (high vs. low concentrations, divided at median in controls; progesterone, testosterone, estrogen, estradiol) using likelihood ratio tests to compare models in- and excluding interaction terms with these factors. For hormone receptor status and age at diagnosis (<50 vs. ≥50 years), we assessed potential heterogeneity using polytomous conditional logistic regression models comparing models assuming the same association versus different associations between sRANKL or the sRANKL/OPG ratio and breast cancer subgroups (e.g., ER+ and ER−; ref. 26). A sensitivity analysis excluding cases diagnosed within 2 years of blood collection ($n = 367$, 19%) was performed to address the possibility of reverse causation.

All statistical tests were two-tailed and considered significant at $P < 0.05$. Statistical analyses were conducted using SAS 9.3 (SAS Institute Inc.).

Reproducibility study

A reproducibility study was conducted in 221 women who were randomly selected from the 592 EPIC-Heidelberg participants who donated blood samples at baseline (1994–1998) and after 14 and 15 years of follow-up. Both the EPIC-Heidelberg cohort and the reproducibility study have been described previously (12, 27). One-year (between 14 and 15 years of follow-up) and 14-year (between baseline and 14 years of follow-up) reproducibility of sRANKL and OPG was assessed using Spearman correlation coefficients. Results for within-person reproducibility for OPG ($r = 0.85$ over 1 year and $r = 0.75$ over 14 years) have been published previously (12).

Results

At blood collection, the majority of the study population (77%) was postmenopausal, and half of the postmenopausal women (758 case–control sets, 50%) were using exogenous hormones (Table 1). Median age at blood collection was 56 years (range 27–77 years), and median age of diagnosis for cases was 61 years (range 35–84 years). Among cases, 81% were ER+ and 19% were ER−.

Adjusting for matching factors, sRANKL concentrations were weakly to moderately inversely correlated with OPG concentrations among study controls (e.g., premenopausal women, Spearman $r = -0.40$). Concentrations of sRANKL were not, or only weakly (Spearman $r < |0.30|$), correlated with age, body mass index (BMI, kg/m^2), or the other evaluated hormones (Supplementary Table S1). With the exception of variation by age and menopausal status at blood collection, the distribution of covariates was similar between sRANKL quintiles for both cases and controls (Supplementary Table S2).

There was suggestive heterogeneity in the association between sRANKL and breast cancer risk by hormone receptor status (ER+PR+ vs. ER−PR− $P_{het} = 0.05$; ER+ vs. ER− $P_{het} = 0.13$; Table 2). sRANKL was suggestively associated with

Table 1. Population characteristics: EPIC nested case-control study

	Cases	Controls
Full study population, *n*	1976[a]	1976[a]
Baseline characteristics, median (range), or *n* (%)		
Age at blood collection, years	56 (27–76)	56 (27–77)
Age at menarche, years	13 (8–20)	13 (8–19)
Premenopausal	460 (23%)	460 (23%)
Postmenopausal	1,516 (77%)	1,516 (77%)
PMH use at blood collection[b]	758 (50%)	758 (50%)
Age at menopause, years[b]	50 (27–63)	50 (21–63)
Completed term pregnancy	1,675 (86%)	1,709 (88%)
Age at first term pregnancy[c], years	25 (16–44)	24 (15–42)
BMI, kg/m^2	24 (14–49)	24 (16–46)
sRANKL concentrations, pmol/L[d]	0.11 (0.005, 1.67)	0.11 (0.005, 0.85)
OPG concentrations, pmol/L[a]	9.81 (2.94, 31.81)	9.84 (3.52, 32.86)
sRANKL/OPG ratio[a]	0.01 (0.0002, 0.17)	0.01 (0.0002, 0.20)
Case characteristics		
ER+	1,598 (81%)	
ER−	378 (19%)	
ER+/PR+[e]	920 (63%)	
ER−/PR−[e]	251 (17%)	
Age at diagnosis, years	61 (35–84)	
Time between blood donation and diagnosis, years	4.7 (0.02–11.7)	

[a]An additional nine case–control sets were missing OPG measurements. The total number of case–control sets for the sRANKL/OPG ratio is $n = 1{,}967$.
[b]Among postmenopausal women.
[c]Among women with completed term pregnancy.
[d]Lowest measured value was 0.01 pmol/L; 327 observations (8.1%; 175 cases, 152 controls) had sRANKL concentrations below the LLOD of the assay, there were set to 50% the LLOD.
[e]PR status available for 74% of cases (sRANKL $n = 1{,}461$, sRANKL/OPG ratio $n = 1{,}454$); percentages represent percentage of total cases with ER and PR status available.

ER+PR+ breast cancer [fifth vs. first quintile RR = 1.36 (95% CI, 0.99–1.87); $P_{trend} = 0.31$] and significantly associated with ER+ disease [fifth vs. first quintile RR = 1.28 (95% CI, 1.01–1.63); $P_{trend} = 0.20$]. We observed no association between sRANKL and hormone receptor negative disease [e.g., ER− fifth vs. first quintile RR = 0.87 (95% CI, 0.53–1.44); $P_{trend} = 0.21$]. There was no heterogeneity by age at diagnosis (<50 vs. ≥50 years $P_{het} \geq 0.52$), however, associations of sRANKL with ER+ and ER+PR+ disease were only observed in women who were diagnosed at an older age [age ≥50 years, fifth vs. first quintile: RR ER+ 1.33 (95% CI, 1.03–1.70); $P_{trend} = 0.22$ and ER+PR+ 1.44 (95% CI, 1.02–2.03); $P_{trend} = 0.33$].

The association between the sRANKL/OPG ratio and breast cancer risk differed significantly by hormone receptor status (ER+PR+ vs. ER−PR− $P_{het} = 0.02$; ER+ vs. ER− $P_{het} = 0.05$). A higher sRANKL/OPG ratio was associated with increased risk of ER+ breast cancer [fifth vs. first quintile RR: ER+ 1.33 (95% CI, 1.03–1.71); $P_{trend} = 0.12$ and ER+PR+ 1.42 (95% CI, 1.01–1.98); $P_{trend} = 0.21$; Table 3). We observed a significant trend suggesting an inverse association between the sRANKL/OPG ratio and ER−PR− disease ($P_{trend} = 0.03$); however, we observed no significant association in the quintile contrast [fifth vs. first quintile RR = 0.60 (0.31–1.14)]. Similar to sRANKL, there was no heterogeneity by age at diagnosis (<50 vs. ≥50 years $P_{het} =$ ≥0.43); however, associations between the sRANKL/OPG ratio and ER+ and ER+PR+ disease remained significant only in those ages ≥50 years at diagnosis [fifth vs. first quintile RR: ER+ 1.34 (95% CI, 1.03, 1.75); $P_{trend} = 0.14$] and ER+PR+ 1.44 (95% CI, 1.00–2.06); $P_{trend} = 0.25$]. We observed no heterogeneity by menopausal status at blood collection (Supplementary Tables S3 and S4).

Associations between sRANKL and breast cancer risk were similar before and after adjusting for OPG concentrations [e.g., fifth vs. first quintile RR ER+PR+: before adjustment: 1.36 (95% CI, 0.99–1.87) and after adjustment: 1.32 (95% CI, 0.94–1.85); Supplementary Table S5]. No associations were seen in analyses considering the cross-classification of sRANKL and OPG concentrations [e.g., high sRANKL and low OPG vs. low sRANKL and high OPG RR: ER+PR+ 1.04 (95% CI, 0.87-1.24); Supplementary Table S6].

Additional adjustment for endogenous hormone concentrations and reproductive and lifestyle factors did not change the interpretation of results. We observed no effect modification by circulating estrogens, progesterone, testosterone, prolactin, smoking status, ever use of OCs or PMH, use of PMH at blood collection, or ever having had a full term pregnancy ($p_{int} \geq$ 0.06). Excluding women diagnosed within 2 years of blood donation in sensitivity analyses did not impact the results (data not shown).

Spearman correlations of sRANKL concentrations over 1 year were $r = 0.60$; correlations between concentrations in samples taken 14 years apart were $r = 0.38$. For the sRANKL/OPG ratio, correlations were $r = 0.69$ over 1 year and $r = 0.48$ over 14 years.

Discussion

This large prospective study is the first large-scale investigation on circulating sRANKL and the sRANKL/OPG ratio and breast cancer risk, and includes detailed analyses by hormone receptor subtype. A higher sRANKL/OPG ratio was associated with significantly higher risk of hormone receptor positive disease, particularly among women diagnosed at older ages. Results for sRANKL concentrations were similar for hormone receptor positive disease. The sRANKL/OPG ratio was inversely associated with hormone receptor negative breast cancer, consistent with our

Table 2. Circulating concentrations of sRANKL and breast cancer risk by hormone-receptor subtype: EPIC nested case–control study

	Quintiles								
	1	2	3	4	5				
Cut-off points (pmol/L)[a]	<0.05	0.05–0.09	0.10–0.14	0.15–0.20	≥0.20	P_{trend}[b]	RRlog2	P_{het}[c]	P_{het}[d]
Whole population									
ER+/PR+									
Cases/controls	167/198	203/183	176/192	160/157	214/190		920/920		
RR (95% CI)	Ref.	1.31 (0.96–1.80)	1.12 (0.81–1.55)	1.18 (0.84–1.64)	1.36 (0.99–1.87)	0.31	1.03 (0.97–1.10)	0.05	
ER+									
Cases/controls	339/364	360/351	301/327	255/264	343/292		1,598/1,598		
RR (95% CI)	Ref.	1.11 (0.89–1.39)	1.02 (0.80–1.29)	1.03 (0.81–1.32)	1.28 (1.01–1.63)	0.20	1.03 (0.98–1.08)	0.13	
ER−/PR−									
Cases/controls	52/51	57/40	47/53	49/56	46/51		251/251		
RR (95% CI)	Ref.	1.54 (0.81–2.93)	0.94 (0.50–1.75)	0.81 (0.43–1.54)	0.74 (0.39–1.38)	0.08	0.89 (0.78–1.01)		
ER−									
Cases/controls	84/79	83/63	73/82	63/85	75/69		378/378		
RR (95% CI)	Ref.	1.23 (0.74–2.03)	0.75 (0.46–1.22)	0.63 (0.37–1.06)	0.87 (0.53–1.44)	0.21	0.94 (0.84–1.04)		
Age at diagnosis <50 years									
ER+/PR+									
Cases/controls	12/12	27/29	18/21	24/15	40/44		121/121		
RR (95% CI)	Ref.	0.68 (0.21–2.24)	0.56 (0.15–2.08)	1.33 (0.37–4.81)	0.90 (0.29–2.80)	0.74	1.04 (0.81–1.34)		
ER+									
Cases/controls	16/15	32/34	21/27	26/18	48/49		143/143		
RR (95% CI)	Ref.	0.64 (0.22–1.81)	0.44 (0.14–1.43)	1.00 (0.32–3.16)	0.78 (0.28–2.19)	0.90	1.02 (0.81–1.27)		
Age at diagnosis ≥50 years									
ER+/PR+									
Cases/controls	155/186	176/154	158/171	136/142	174/146		799/799		
RR (95% CI)	Ref.	1.38 (0.99–1.92)	1.17 (0.84–1.64)	1.13 (0.79–1.60)	1.44 (1.02–2.03)	0.33	1.03 (0.97–1.10)	0.05	0.81
ER+									
Cases/controls	323/349	328/317	280/300	229/246	295/243		1,455/1,455		
RR (95% CI)	Ref.	1.13 (0.90–1.43)	1.05 (0.82–1.34)	1.00 (0.78–1.29)	1.33 (1.03–1.70)	0.22	1.03 (0.98–1.08)	0.12	0.90
ER−/PR−									
Cases/controls	51/47	49/34	41/49	42/45	34/42		217/217		
RR (95% CI)	Ref.	1.51 (0.75–3.03)	0.84 (0.43–1.64)	0.84 (0.42–1.69)	0.57 (0.28–1.13)	0.06	0.88 (0.77–1.01)		0.53
ER−									
Cases/controls	79/74	72/52	60/71	53/68	55/54		319/319		
RR (95% CI)	Ref.	1.37 (0.79–2.36)	0.75 (0.45–1.25)	0.70 (0.40–1.23)	0.77 (0.44–1.33)	0.19	0.93 (0.83–1.04)		0.52

[a]Cutpoints reflect non-log transformed sRANKL concentrations.

[b]P_{trend} based on log2-transformed sRANKL concentrations.

[c]$P_{heterogeneity}$ comparing ER+/PR+ to ER−/PR− and ER+ to ER− subtypes, based on RRlog2.

[d]$P_{heterogeneity}$ comparing age at diagnosis <50 to ≥50 years based on RRlog2.

Conditional logistic regression models adjusted for: ages at menarche (<12, 13, 14, ≥15, missing), menopause (<44, 44–47, 48–50, 51–52, 53–54, ≥55, missing), and first full-term pregnancy (no FTP, <25, 25–30, ≥30, missing), and number of full-term pregnancies (0, 1, 2, ≥3, missing) and BMI (kg/m^2, continuous).

Table 3. The sRANKL/OPG ratio and breast cancer risk by hormone receptor subtype: EPIC nested case–control study

	Quintiles								
	1	2	3	4	5				
Cut-off points[a]	<0.003	0.003–0.008	0.008–0.014	0.014–0.0226	≥0.0226	P_{trend}[b]	RRlog2	p_{het}[c]	p_{het}[d]
Whole population									
ER+/PR+									
Cases/controls	146/177	175/181	186/178	184/180	224/199		915/915		
RR (95% CI)	Ref.	1.17 (0.84–1.63)	1.26 (0.90–1.76)	1.21 (0.86–1.69)	1.42 (1.01–1.98)	0.21	1.04 (0.98–1.10)	0.02	
ER+									
Cases/controls	296/328	332/333	311/318	301/306	350/305		1,590/1,590		
RR (95% CI)	Ref.	1.13 (0.90–1.43)	1.12 (0.88–1.42)	1.10 (0.86–1.40)	1.33 (1.03–1.71)	0.12	1.03 (0.99–1.08)	0.05	
ER−/PR−									
Cases/controls	45/41	46/42	53/47	60/60	46/60		250/250		
RR (95% CI)	Ref.	1.10 (0.56–2.15)	1.04 (0.55–1.98)	0.86 (0.44–1.66)	0.60 (0.31–1.14)	0.03	0.88 (0.78–0.99)		
ER−									
Cases/controls	70/66	70/60	80/76	79/87	78/88		377/377		
RR (95% CI)	Ref.	1.24 (0.73–2.09)	0.93 (0.56–1.55)	0.77 (0.46–1.31)	0.74 (0.44–1.25)	0.10	0.92 (0.84–1.01)		
Age at diagnosis <50 years									
ER+/PR+									
Cases/controls	7/10	20/20	17/25	28/18	48/47		120/120		
RR (95% CI)	Ref.	0.65 (0.16–2.74)	0.36 (0.07–1.74)	1.14 (0.29–4.51)	0.94 (0.25–3.58)	0.48	1.08 (0.87–1.35)		
ER+									
Cases/controls	11/12	23/24	21/29	31/24	56/53		142/142		
RR (95% CI)	Ref.	0.58 (0.16–2.05)	0.36 (0.10–1.36)	0.79 (0.24–2.59)	0.74 (0.23–2.41)	0.67	1.04 (0.85–1.28)		
Age at diagnosis ≥50 years									
ER+/PR+									
Cases/controls	139/167	155/161	169/153	156/162	176/152		795/795		
RR (95% CI)	Ref.	1.18 (0.84–1.66)	1.37 (0.97–1.94)	1.13 (0.80–1.61)	1.44 (1.00–2.06)	0.25	1.04 (0.97–1.10)	0.02	0.98
ER+									
Cases/controls	285/316	309/309	290/289	270/282	294/252		1,448/1,448		
RR (95% CI)	Ref.	1.14 (0.90–1.45)	1.17 (0.91–1.51)	1.06 (0.82–1.37)	1.34 (1.03–1.75)	0.14	1.03 (0.99–1.08)	0.05	0.97
ER−/PR−									
Cases/controls	44/38	43/38	47/41	51/55	31/44		216/216		
RR (95% CI)	Ref.	1.13 (0.56–2.28)	1.06 (0.53–2.10)	0.78 (0.38–1.57)	0.47 (0.23–0.98)	0.03	0.87 (0.76–0.98)		0.43
ER−									
Cases/controls	67/62	64/55	68/61	66/75	53/65		318/318		
RR (95% CI)	Ref.	1.22 (0.70–2.13)	1.03 (0.60–1.78)	0.74 (0.42–1.29)	0.65 (0.37–1.15)	0.10	0.92 (0.83–1.02)		0.50

[a]Cut-off points reflect non-log transformed sRANKL/OPG ratio values.

[b]P_{trend} based on log2-transformed sRANKL/OPG ratio.

[c]$P_{heterogeneity}$ comparing ER+/PR+ to ER−/PR− and ER+ to ER− subtypes, based on RRlog2.

[d]$P_{heterogeneity}$ comparing age at diagnosis <50 to ≥50 years based on RRlog2.

Conditional logistic regression models adjusted for: ages at menarche (<12, 13, 14, ≥15, missing), menopause (<44, 44–47, 48–50, 51–52, 53–54, ≥55, missing), and first full-term pregnancy (no FTP, <25, 25–30, ≥30, missing), and number of full-term pregnancies (0, 1, 2, ≥3, missing) and BMI (kg/m^2, continuous).

previous finding of a positive association between OPG concentrations and hormone receptor negative breast cancer (12).

In humans, RANKL protein or mRNA expression in normal breast tissue is higher in relatively high progesterone conditions—i.e., during luteal phase of the menstrual cycle and during pregnancy (10, 11). In experimental models, *RANKL* expression in mammary cells of ovariectomized mice was elevated in both luminal and MaSC-enriched basal cells following injection of 17B-estradiol and progesterone, but not after injection of progesterone only (28). Similarly, progesterone injection strongly induced RANKL mRNA and protein expression in mammary tissue of non-ovariectomized, nonpregnant, nulliparous mice (i.e., in the presence of natural estrogens; ref. 3). In addition, expression of both RANKL mRNA and protein in mice is induced by both prolactin and parathyroid hormone protein-related peptide (3) and *RANKL* expression is higher in luminal mammary cells of pregnant, as compared to virgin, mice (29).

In contrast, *RANK* expression was abundant in mouse mammary stem cells both mid-pregnancy and following 17ß-estradiol plus progesterone treatment in ovariectomized mice (28, 29). Treatment of mouse mammary stem cells and luminal cells with RANK-Fc, a RANKL antagonist, inhibited clonogenic activity of mouse mammary stem cells but not luminal cells (29). This is consistent with paracrine effects of RANK signaling, with progesterone inducing RANKL expression by luminal cells in the breast, which binds to RANK expressed on mammary stem cells.

Both the absence of *RANK* and absence of overexpression of *RANKL* in mouse models result in nonfunctional mammary glands (3). Elongation of the ductal tree and side branching occur as normal in the mammary gland of *RANKL*-deficient mice; however, alveolar differentiation and maturation are significantly impaired due to defective proliferation and increased apoptosis (3). Overexpression of *RANKL* in the virgin mouse mammary gland is sufficient to trigger side branching in the absence of PR (7, 30, 31).

Aside from the role of the RANK-axis in the normal mammary gland, experimental data suggest a role in mammary carcinogenesis (8, 9). *RANK* expression has been shown to play a role in metastasis of primary breast and prostate cancer to sources of RANKL such as bone (4, 5, 32). *RANK*, *RANKL*, and *OPG* are expressed in a number of breast cancer cells lines and primary breast tumors (32–35), and expression of RANK protein or mRNA has been associated with higher cancer grade, hormone receptor negative/basal like tumors, and a shorter overall and bone metastasis free survival (36–39). *RANKL* expression in breast tumors has been linked to metastasis (33, 36). Tumors expressing OPG protein or mRNA, the decoy receptor for RANKL which prevents RANKL binding to RANK, correlate with lower tumor grade, longer overall and disease-free survival (37, 40). This has not been universally observed, one study found lower tumor *RANK* and *RANKL* expression and higher tumor *OPG* expression to be associated with worse clinical outcomes (33), and one study observed an association between higher serum OPG expression and burden of metastatic disease (41).

Denosumab is a human monoclonal antibody that mimics the effect of OPG and inhibits binding of RANKL to RANK (42). It has been approved by the FDA for treatment of osteoporosis in postmenopausal women and prevention of skeletal-related events in patients with bone metastases from solid tumors (16, 42, 43). It has also been shown to prevent bone loss in breast cancer patients treated with aromatase inhibitors (44). A phase III trial in early breast cancer patients at high risk of recurrence (NCT01077154) is currently underway. Although denosumab delayed time to first fracture (45), outcomes relating to bone metastases and survival are yet to be reported. Breast tissue from BRCA1 carriers has been shown to be hyper-responsive to progesterone and inhibition of RANKL using denosumab has been shown to attenuate progesterone induced epithelial cells proliferation (Ki67 expression) in these tissues (17).

Epidemiologic data on the RANK-axis and breast cancer risk are sparse, with only one previous study evaluating circulating concentrations of sRANKL and ER+ breast cancer risk (15) and three previous investigations, including our own, evaluating circulating OPG and disease risk in the general population (12, 13, 15) and one in BRCA mutation carriers (14). Following experimental evidence, the hypothesized role of denosumab in breast cancer patients, and previous data on circulating RANK-axis member OPG and breast cancer, we hypothesized a positive association between sRANKL, and the sRANKL/OPG ratio, and breast cancer risk. In this first large-scale prospective study, we observed limited evidence for an association between sRANKL, or the sRANKL/OPG ratio, and breast cancer risk, with an indication that higher sRANKL or sRANKL/OPG ratio may be associated with higher risk of hormone receptor-positive disease. In line with our prior study, in which we observed a positive association between OPG and hormone-receptor negative breast cancer (12), the sRANKL/OPG ratio was inversely associated with hormone-receptor negative disease risk in this study. Results were similar in analyses adjusting for or stratifying by endogenous hormone concentrations or exogenous hormone use and menopausal status at blood collection. Although no statistically significant heterogeneity was seen by age of diagnosis, associations with breast cancer, similar in magnitude to those observed in the whole population, remained only among those aged >50 years at diagnosis (evaluated as a proxy for menopausal status).

The literature on RANK/RANKL signaling in breast development and carcinogenesis predominantly focuses on paracrine signaling in the breast, with only few studies reporting results on effects of circulating concentrations. One study found that inhibition of RANKL using a monoclonal antibody (OPG-Fc) reduces colony formation of estrogen and PR negative cells expressing *RANK*, but not colony formation of hormone receptor positive cells of young adult mice (17). Similarly, injection of recombinant RANKL compared to control injection led to increased proliferation of mammary epithelial cells in mice lacking *PR*, which was in turn inhibited by injection of OPG (30). Extending these findings to a BRCA1 mouse model, treatment with OPG-Fc delayed tumor onset compared to the control treatment (17).

It is plausible that circulating sRANKL concentrations are not representative of concentrations in the breast tissue itself, and concentrations in the normal breast are a more informative measure. Although it is known that progesterone and prolactin are associated with RANKL expression in the breast, we saw no correlation between circulating concentrations of these hormones and sRANKL. To our knowledge, the association between circulating and breast tissue RANKL in humans has not previously been described. However, one prior study observed higher mammary *RANKL* in macaques treated with estrogen plus progestin, relative to control animals, whereas serum RANKL concentrations were similar in both groups (46). In contrast, both mammary and serum OPG were lower in the estrogen plus progestin treatment

group, relative to controls. An additional limitation of our study is the use of a single measurement of sRANKL to characterize exposure. We observed moderate correlation ($r = 0.60$) between sRANKL measurements in samples taken 1-year apart, which is similar to previously reported correlations over 5 years ($r = 0.63$; ref. 47). Correlations between sRANKL concentrations in samples taken 14 years study were relatively low. Correlations for the sRANKL/OPG ratio were somewhat stronger. This relatively low within-person reproducibility for sRANKL suggests one measure may not represent longer-term exposure and would result in nondifferential misclassification, and an attenuation of the relative risk. In addition, the majority of RANKL in the human body is cell bound and not detectable in circulation (48). We observed relatively low sRANKL concentrations overall, and 8.3% ($n = 327$) of the study population had concentrations below the limit of detection. In addition, previous work on RANKL and breast cancer has focused on BRCA-mutation carriers. We were unable to restrict our analyses to a high-risk population as BRCA-status is unavailable in the EPIC cohort and information on family history of breast cancer is limited (61% missing, 4% reporting a positive family history). Further, we observed inter-batch CVs of 21.7%, reflecting measurement error, for OPG in postmenopausal women. This may have led to nondifferential misclassification and attenuation of results. We observed relatively low concentrations of OPG, as compared to others (13, 14); however, this difference in absolute concentrations would not impact the relative ranking of participants in quintiles. Finally, although the number of cases included was large, only a limited number were diagnosed at a younger age, preventing us from evaluating risk of hormone receptor negative breast cancer in younger women (<50 years at diagnosis).

Conclusion

RANK-axis has been widely discussed as a potential target for breast cancer prevention (49) and, with the fully human antibody denosumab showing benefit for cancer patients in clinical trials there is increasing interest in RANKL as a target for prevention and treatment of breast cancer. However, this first large-scale investigation on circulating sRANKL in women provides only limited support for a role for circulating sRANKL in breast cancer risk. Further investigations in large, well-characterized populations are needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Grant Support

This project was funded by research grant no. 111454 from the Deutsche Kresbshilfe. R.T. Fortner was supported by a Marie Curie International Incoming Fellowship of the European Commission's Seventh Framework Programme (MC-IIF-623984).

The coordination of EPIC is financially supported by the European Commission (DG-SANCO) and the IARC. The national cohorts are supported by Danish Cancer Society (Denmark); Ligue Contre le Cancer, Institut Gustave Roussy, Mutuelle Générale de l'Education Nationale, Institut National de la Santé et de la Recherche Médicale (INSERM) (France); German Cancer Aid, DKFZ, Federal Ministry of Education and Research (BMBF), Deutsche Krebshilfe, Deutsches Krebsforschungszentrum, and Federal Ministry of Education and Research (Germany); the Hellenic Health Foundation (Greece); Associazione Italiana per la Ricerca sul Cancro-AIRC-Italy and National Research Council (Italy); Dutch Ministry of Public Health, Welfare and Sports (VWS), the Netherlands Cancer Registry (NKR), LK Research Funds, Dutch Prevention Funds, Dutch ZON (Zorg Onderzoek Nederland), World Cancer Research Fund (WCRF), Statistics Netherlands (The Netherlands); ERC-2009-AdG 232997 and Nordforsk, Nordic Centre of Excellence programme on Food, Nutrition and Health (Norway); Health Research Fund (FIS), PI13/00061 to Granada; PI13/01162 to EPIC-Murcia), Regional Governments of Andalucía, Asturias, Basque Country, Murcia and Navarra, ISCIII RETIC (RD06/0020) (Spain); Swedish Cancer Society, Swedish Research Council and County Councils of Skåne and Västerbotten (Sweden); Cancer Research UK (14136 to EPIC-Norfolk; C570/A16491 and C8221/A19170 to EPIC-Oxford), Medical Research Council (1000143 to EPIC-Norfolk, MR/M012190/1 to EPIC-Oxford; United Kingdom).

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Received April 26, 2017; revised June 14, 2017; accepted June 26, 2017; published OnlineFirst July 12, 2017.

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