

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



Danja Sarink¹, Helena Schock¹, Theron Johnson¹, Kim Overvad², Marianne Holm³, Anne Tjønneland³, Marie-Christine Boutron-Ruault^{4,5}, Mathilde His^{4,5}, Marina Kvaskoff^{4,5}, Heiner Boeing⁶, Pagona Lagiou^{7,8,9}, Eleni-Maria Papatesta⁷, Antonia Trichopoulou^{7,8}, Domenico Palli¹⁰, Valeria Pala¹¹, Amalia Mattiello¹², Rosario Tumino¹³, Carlotta Sacerdote¹⁴, H.B(as). Bueno-de-Mesquita^{15,16}, Carla H. van Gils¹⁷, Petra H. Peeters^{17,18}, Elisabete Weiderpass^{19,20,21,22}, Antonio Agudo²³, Maria-José Sánchez^{24,25}, Maria-Dolores Chirlaque^{25,26,27}, Eva Ardanaz^{25,28,29}, Pilar Amiano^{25,30}, Kay Tee Khaw³¹, Ruth Travis³², Laure Dossus³³, Mark Gunter³³, Sabina Rinaldi³³, Melissa Merritt¹⁶, Elio Riboli¹⁶, Rudolf Kaaks¹, and Renée T. Fortner¹

Abstract

Receptor activator of nuclear factor-kappa B (RANK)-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.

¹Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany. ²Department of Public Health, Section for Epidemiology, Aarhus University, Aarhus, Denmark. ³Unit of Diet, Genes and Environment, Danish Cancer Society Research Center, Copenhagen, Denmark. ⁴Université Paris-Saclay, Université Paris-Sud, UVSQ, CESP, INSERM, Villejuif, France. ⁵Gustave Roussy, Villejuif, France. ⁶Department of Epidemiology, German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany. ⁷Hellenic Health Foundation, Athens, Greece. ⁸WHO Collaborating Center for Nutrition and Health, Unit of Nutritional Epidemiology and Nutrition in Public Health, Department of Hygiene, Epidemiology and Medical Statistics, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece. ⁹Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts. ¹⁰Cancer Risk Factors and Lifestyle Epidemiology Unit, Cancer Research and Prevention Institute, ISPO, Florence, Italy. ¹¹Epidemiology and Prevention Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy. ¹²Dipartimento di Medicina Clinica e Chirurgia, Federico II University, Naples, Italy. ¹³Cancer Registry and Histopathology Department, "Civic- M.P Arezzo" Hospital, ASP Ragusa, Italy. ¹⁴Unit of Cancer Epidemiology, Città della Salute e della Scienza University-Hospital and Center for Cancer Prevention (CPO), Turin, Italy. ¹⁵Department for Determinants of Chronic Diseases (DCD), National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands. ¹⁶Department of Epidemiology and Biostatistics, The School of Public Health, Imperial College London, London, United Kingdom. ¹⁷Department of Epidemiology, Julius Center for Health Sciences and Primary Care, University Medical Center, Utrecht, the Netherlands. ¹⁸MRC-PHE Centre for Environment and Health, Department of Epidemiology and Biostatistics, School of Public Health, Imperial College, London, United Kingdom. ¹⁹Department of Community Medicine, Faculty of Health Sciences, University of Tromsø, The Arctic University of Norway, Tromsø,

Norway. ²⁰Department of Research, Cancer Registry of Norway, Institute of Population-Based Cancer Research, Oslo, Norway. ²¹Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden. ²²Genetic Epidemiology Group, Folkhälsan Research Center, Helsinki, Finland. ²³Unit of Nutrition and Cancer, IDIBELL, Catalan Institute of Oncology (ICO), L'Hospitalet de Llobregat, Barcelona, Spain. ²⁴Escuela Andaluza de Salud Pública. Instituto de Investigación Biosanitaria ibs.Granada. Hospitales Universitarios de Granada/Universidad de Granada, Granada, Spain. ²⁵CIBER de Epidemiología y Salud Pública (CIBERESP), Madrid, Spain. ²⁶Department of Epidemiology, Regional Health Council, IMIB-Arrixaca, Murcia, Spain. ²⁷Department of Health and Social Sciences, Universidad de Murcia, Murcia, Spain. ²⁸Navarra Public Health Institute, Pamplona, Spain. ²⁹IdiSNA, Navarra Institute for Health Research, Pamplona, Spain. ³⁰Public Health Division of Gipuzkoa, BioDonostia Health Research Institute, San Sebastian, Spain. ³¹Cancer Epidemiology Unit, University of Cambridge, Cambridge, United Kingdom. ³²Cancer Epidemiology Unit, Nuffield Department of Population Health, University of Oxford, Oxford, United Kingdom. ³³International Agency for Research on Cancer, Lyon, France.

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Corresponding Author: Renée T. Fortner, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. Phone: +49 6221 42 2241; Fax: +49 6221 42 2203; E-mail: r.fortner@dkfz.de

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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 lobulo-alveolar 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); $P_{\text{trend}} = 0.03$ and ER+ 0.84 (95% CI, 0.68–1.04); $P_{\text{trend}} = 0.18$. $n = 2,008$ 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 (LOD; 0.01 pmol/L).

Statistical analyses

Concentrations of sRANKL and OPG (both 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- $P_{\text{het}} = 0.05$; ER+ vs. ER- $P_{\text{het}} = 0.13$; Table 2). sRANKL was suggestively associated with

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

	Cases	Controls
Full study population, <i>n</i>	1976 ^a	1976 ^a
Baseline characteristics, median (range), or <i>n</i> (%)		
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)	

^aAn 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.

^bAmong postmenopausal women.

^cAmong women with completed term pregnancy.

^dLowest 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.

^ePR 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); $P_{\text{trend}} = 0.31$] and significantly associated with ER+ disease [fifth vs. first quintile RR = 1.28 (95% CI, 1.01–1.63); $P_{\text{trend}} = 0.20$]. 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); $P_{\text{trend}} = 0.21$]. There was no heterogeneity by age at diagnosis (<50 vs. ≥50 years $P_{\text{het}} \geq 0.52$), 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); $P_{\text{trend}} = 0.22$ and ER+PR+ 1.44 (95% CI, 1.02–2.03); $P_{\text{trend}} = 0.33$].

The association between the sRANKL/OPG ratio and breast cancer risk differed significantly by hormone receptor status (ER+PR+ vs. ER–PR– $P_{\text{het}} = 0.02$; ER+ vs. ER– $P_{\text{het}} = 0.05$). 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); $P_{\text{trend}} = 0.12$ and ER+PR+ 1.42 (95% CI, 1.01–1.98); $P_{\text{trend}} = 0.21$; Table 3]. We observed a significant trend suggesting an inverse association between the sRANKL/OPG ratio and ER–PR– disease ($P_{\text{trend}} = 0.03$); 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 $P_{\text{het}} = \geq 0.43$); 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); $P_{\text{trend}} = 0.14$] and ER+PR+ 1.44 (95% CI, 1.00–2.06); $P_{\text{trend}} = 0.25$]. 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 ($p_{\text{int}} \geq 0.06$). 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					RRlog2	P _{trend} ^b	P _{net} ^c	P _{net} ^d
	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				
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
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
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

^aCutpoints reflect non-log transformed sRANKL concentrations.

^bP_{trend} based on log2-transformed sRANKL concentrations.

^cP_{heterogeneity} comparing ER+/PR+ to ER-/PR- and ER+ to ER- subtypes, based on RRlog2.

^dP_{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/m², continuous).

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

	Quintiles					RRlog2	P _{trend} ^b	P _{het} ^c	P _{het} ^d
	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				
Whole population									
ER+/PR+									
Cases/controls	146/177	175/181	186/178	184/180	224/199	915/915	0.21	0.02	
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)	1.04 (0.98-1.10)			
ER+									
Cases/controls	296/328	332/333	311/318	301/306	350/305	1,590/1,590	0.12	0.05	
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)	1.03 (0.99-1.08)			
ER-/PR-									
Cases/controls	45/41	46/42	53/47	60/60	46/60	250/250	0.03		
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)	0.88 (0.78-0.99)			
ER-									
Cases/controls	70/66	70/60	80/76	79/87	78/88	377/377	0.10		
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)	0.92 (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	0.48		
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)	1.08 (0.87-1.35)			
ER+									
Cases/controls	11/12	23/24	21/29	31/24	56/53	142/142	0.67		
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)	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	0.25	0.02	0.98
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)	1.04 (0.97-1.10)			
ER+									
Cases/controls	285/316	309/309	290/289	270/282	294/252	1,448/1,448	0.14	0.05	0.97
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)	1.03 (0.99-1.08)			
ER-/PR-									
Cases/controls	44/38	43/38	47/41	51/55	31/44	216/216	0.03		0.43
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)	0.87 (0.76-0.98)			
ER-									
Cases/controls	67/62	64/55	68/61	66/75	53/65	318/318	0.10		0.50
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)	0.92 (0.83-1.02)			

^aCut-off points reflect non-log transformed sRANKL/OPG ratio values.

^bP_{trend} based on log2-transformed sRANKL/OPG ratio.

^cP_{het} heterogeneity comparing ER+/PR+ to ER-/PR- and ER+ to ER- subtypes, based on RRlog2.

^dP_{het} 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/m², 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 17 β -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 17 β -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 cell 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.

Authors' Contributions

Conception and design: T. Johnson, K. Overvad, A. Tjønneland, H. Boeing, R. Tumino, H.B(as). Bueno-de-Mesquita, P.H. Peeters, E. Weiderpass, E. Ardanaz, R. Kaaks, R.T. Fortner

References

1. Boyce BF, Xing L. Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Res Thera* 2007;9:S1.
2. Wada T, Nakashima T, Hiroshi N, Penninger JM. RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* 2006;12:17–25.
3. Fata JE, Kong YY, Li J, Sasaki T, Irie-Sasaki J, Moorehead RA, et al. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* 2000;103:41–50.

Development of methodology: T. Johnson, H.B(as). Bueno-de-Mesquita, E. Weiderpass, M.-D. Chirlaque, E. Ardanaz

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Johnson, K. Overvad, A. Tjønneland, M.-C. Boutron-Ruault, H. Boeing, P. Lagiou, A. Trichopoulou, D. Palli, V. Pala, A. Mattiello, R. Tumino, C. Sacerdote, H.B(as). Bueno-de-Mesquita, P.H. Peeters, E. Weiderpass, A. Agudo, M.-J. Sánchez, M.-D. Chirlaque, E. Ardanaz, P. Amiano, K.T. Khaw, R. Travis, R. Kaaks

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Sarink, H. Schock, T. Johnson, M. Holm, M. Kvaskoff, D. Palli, C.H. van Gils, E. Weiderpass, E. Ardanaz, M. Gunter, R. Kaaks, R.T. Fortner

Writing, review, and/or revision of the manuscript: D. Sarink, H. Schock, T. Johnson, K. Overvad, M. Holm, A. Tjønneland, M.-C. Boutron-Ruault, M. His, M. Kvaskoff, H. Boeing, P. Lagiou, E.-M. Papatesta, A. Trichopoulou, D. Palli, V. Pala, A. Mattiello, R. Tumino, C. Sacerdote, H.B(as). Bueno-de-Mesquita, C.H. van Gils, P.H. Peeters, E. Weiderpass, A. Agudo, M.-J. Sánchez, M.-D. Chirlaque, E. Ardanaz, P. Amiano, K.T. Khaw, R. Travis, L. Dossus, M. Gunter, S. Rinaldi, M. Merritt, E. Riboli, R. Kaaks, R.T. Fortner

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Overvad, C. Sacerdote, P.H. Peeters, E. Weiderpass, A. Agudo, M.-J. Sánchez, E. Ardanaz, K.T. Khaw, R. Kaaks

Study supervision: R. Tumino, P.H. Peeters, E. Weiderpass, A. Agudo, M.-J. Sánchez, E. Ardanaz, R.T. Fortner

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4. Gonzalez-Suarez E, Branstetter D, Armstrong A, Dinh H, Blumberg H, Dougall WC. RANK overexpression in transgenic mice with mouse mammary tumor virus promoter-controlled RANK increases proliferation and impairs alveolar differentiation in the mammary epithelia and disrupts lumen formation in cultured epithelial acini. *Mol Cell Biol* 2007;27:1442–54.
5. Jones DH, Nakashima T, Sanchez OH, Koziarzki I, Komarova SV, Sarosi I, et al. Regulation of cancer cell migration and bone metastasis by RANKL. *Nature* 2006;440:692–6.

6. Mulac-Jericovic B, Lydon JP, DeMayo FJ, Conneely OM. Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. *Proc Natl Acad Sci U S A* 2003;100:9744–9.
7. Fernandez-Valdivia R, Mukherjee A, Ying Y, Li J, Paquet M, DeMayo FJ, et al. The RANKL signaling axis is sufficient to elicit ductal side-branching and alveologenesis in the mammary gland of the virgin mouse. *Dev Biol* 2009;328:127–39.
8. Schramek D, Leibbrandt A, Sigl V, Kenner L, Pospisilik JA, Lee HJ, et al. Osteoclast differentiation factor RANKL controls development of progesterin-driven mammary cancer. *Nature* 2010;468:98–102.
9. Gonzalez-Suarez E, Jacob AP, Jones J, Miller R, Roudier-Meyer MP, Erwert R, et al. RANK ligand mediates progesterin-induced mammary epithelial proliferation and carcinogenesis. *Nature* 2010;468:103–7.
10. Tanos T, Sfomlos G, Echeverria PC, Ayyanan A, Gutierrez M, Delaloye JF, et al. Progesterone/RANKL is a major regulatory axis in the human breast. *Sci Translat Med* 2013;5:182ra55.
11. Wang J, Gupta A, Hu H, Chatterton RT, Clevenger CV, Khan SA. Comment on "Progesterone/RANKL is a major regulatory axis in the human breast." *Sci Translat Med* 2013;5:215le4.
12. Fortner RT, Sarink D, Schock H, Johnson T, Tjonneland A, Olsen A, et al. Osteoprotegerin and breast cancer risk by hormone receptor subtype: a nested case-control study in the EPIC cohort. *BMC Med* 2017;15:26–35.
13. Vik A, Brodin EE, Mathiesen EB, Brox J, Jorgensen L, Njolstad I, et al. Serum osteoprotegerin and future risk of cancer and cancer-related mortality in the general population: the Tromso study. *Eur J Epidemiol* 2015;30:219–30.
14. Oden L, Akbari M, Zaman T, Singer CF, Sun P, Narod SA, et al. Plasma osteoprotegerin and breast cancer risk in BRCA1 and BRCA2 mutation carriers. *Oncotarget* 2016;7:86687–94.
15. Kiehl S, Schramek D, Widschwendter M, Fourkala EO, Zaikin A, Jones A, et al. Aberrant regulation of RANKL/OPG in women at high risk of developing breast cancer. *Oncotarget* 2017;8:3811–25.
16. Stopeck AT, Lipton A, Body JJ, Steger GG, Tonkin K, de Boer RH, et al. Denosumab compared with zoledronic acid for the treatment of bone metastases in patients with advanced breast cancer: a randomized, double-blind study. *J Clin Oncol* 2010;28:5132–9.
17. Nolan E, Vaillant F, Branstetter D, Pal B, Giner G, Whitehead L. RANK ligand as a potential target for breast cancer prevention in BRCA1-mutation carriers. *Nat Med* 2016;22:933–9.
18. Riboli E, Hunt KJ, Slimani N, Ferrari P, Norat T, Fahey M, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. *Public Health Nutr* 2002;5:1113–24.
19. Tikk K, Sookthai D, Johnson T, Rinaldi S, Romieu I, Tjonneland A, et al. Circulating prolactin and breast cancer risk among pre- and postmenopausal women in the EPIC cohort. *Ann Oncol* 2014;25:1422–8.
20. James RE, Lukanova A, Dossus L, Becker S, Rinaldi S, Tjonneland A, et al. Postmenopausal serum sex steroids and risk of hormone receptor-positive and -negative breast cancer: a nested case-control study. *Cancer Prev Res* 2011;4:1626–35.
21. Kaaks R, Tikk K, Sookthai D, Schock H, Johnson T, Tjonneland A, et al. Premenopausal serum sex hormone levels in relation to breast cancer risk, overall and by hormone receptor status - results from the EPIC cohort. *Int J Cancer* 2014;134:1947–57.
22. Kaaks R, Johnson T, Tikk K, Sookthai D, Tjonneland A, Roswall N, et al. Insulin-like growth factor I and risk of breast cancer by age and hormone receptor status—a prospective study within the EPIC cohort. *Int J Cancer* 2014;134:2683–90.
23. Verheus M, Peeters PHM, Rinaldi S, Dossus L, Biessy C, Olsen A, et al. Serum C-peptide levels and breast cancer risk: Results from the European prospective investigation into cancer and nutrition (EPIC). *Int J Cancer* 2006;119:659–67.
24. Kuhn T, Kaaks R, Teucher B, Hirche F, Dierkes J, Weikert C, et al. Dietary, lifestyle, and genetic determinants of vitamin D status: a cross-sectional analysis from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Germany study. *Eur J Nutr* 2014;53:731–41.
25. Rosner B. Percentage Points for a Generalized ESD Many-Outlier Procedure. *Technometrics* 1983;25:165–72.
26. Wang M, Spiegelman D, Kuchiba A, Lochhead P, Kim S, Chan AT, et al. Statistical methods for studying disease subtype heterogeneity. *Stat Med* 2016;35:782–800.
27. Barth SD, Schulze JJ, Kuhn T, Raschke E, Husing A, Johnson T, et al. Treg-Mediated Immune Tolerance and the Risk of Solid Cancers: Findings From EPIC-Heidelberg. *J Natl Cancer Inst* 2015;107:djv224.
28. Joshi PA, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL, et al. Progesterone induces adult mammary stem cell expansion. *Nature* 2010;465:803–7.
29. Asselin-Labat ML, Vaillant F, Sheridan JM, Pal B, Wu D, Simpson ER, et al. Control of mammary stem cell function by steroid hormone signalling. *Nature* 2010;465:798–802.
30. Beleut M, Rajaram RD, Caikovski M, Ayyanan A, Germano D, Choi Y, et al. Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland. *Proc Natl Acad Sci U S A* 2010;107:2989–94.
31. Mukherjee A, Soyak SM, Li J, Ying Y, He B, DeMayo FJ, et al. Targeting RANKL to a specific subset of murine mammary epithelial cells induces ordered branching morphogenesis and alveologenesis in the absence of progesterone receptor expression. *FASEB J* 2010;24:4408–19.
32. Tan W, Zhang W, Strasner A, Grivennikov S, Cheng JQ, Hoffman RM, et al. Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL-RANK signalling. *Nature* 2011;470:548–53.
33. Owen S, Ye L, Sanders AJ, Mason MD, Jiang WG. Expression profile of receptor activator of nuclear-kappaB (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) in breast cancer. *Anticancer Res* 2013;33:199–206.
34. Weichhaus M, Chung ST, Connelly L. Osteoprotegerin in breast cancer: beyond bone remodeling. *Mol Cancer* 2015;14:117–23.
35. Pfitzner BM, Branstetter D, Loibl S, Denkert C, Lederer B, Schmitt WD, et al. RANK expression as a prognostic and predictive marker in breast cancer. *Breast Cancer Res Treat* 2014;145:307–15.
36. Palafox M, Ferrer I, Pellegrini P, Vila S, Hernandez-Ortega S, Urruticoechea A, et al. RANK induces epithelial-mesenchymal transition and stemness in human mammary epithelial cells and promotes tumorigenesis and metastasis. *Cancer Res* 2012;72:2879–88.
37. Santini D, Schiavon G, Vincenzi B, Gaeta L, Pantano F, Russo A, et al. Receptor activator of NF- κ B (RANK) expression in primary tumors associates with bone metastasis occurrence in breast cancer patients. *PLoS One* 2011;6:e19234.
38. Zhang L, Teng Y, Zhang Y, Liu J, Xu L, Qu J, et al. Receptor activator for nuclear factor kappa B expression predicts poor prognosis in breast cancer patients with bone metastasis but not in patients with visceral metastasis. *J Clin Pathol* 2012;65:36–40.
39. Ibrahim T, Sacanna E, Gaudio M, Mercatali L, Scarpi E, Zoli W, et al. Role of RANK, RANKL, OPG, and CXCR4 tissue markers in predicting bone metastases in breast cancer patients. *Clin Breast Cancer* 2011;11:369–75.
40. Holen I, Cross SS, Neville-Webbe HL, Cross NA, Balasubramanian SP, Croucher PI, et al. Osteoprotegerin (OPG) expression by breast cancer cells in vitro and breast tumours in vivo—a role in tumour cell survival? *Breast Cancer Res Treat* 2005;92:207–15.
41. Mountzios G, Dimopoulos MA, Bamias A, Papadopoulos G, Kastiris E, Syrigos K, et al. Abnormal bone remodeling process is due to an imbalance in the receptor activator of nuclear factor-kappaB ligand (RANKL)/osteoprotegerin (OPG) axis in patients with solid tumors metastatic to the skeleton. *Acta Oncologica* 2007;46:221–9.
42. Bone HG, Bolognese MA, Yuen CK, Kendler DL, Miller PD, Yang YC, et al. Effects of denosumab treatment and discontinuation on bone mineral density and bone turnover markers in postmenopausal women with low bone mass. *J Clin Endocrinol Metab* 2011;96:972–80.
43. Fizazi K, Carducci M, Smith M, Damião R, Brown J, Karsh L, et al. Denosumab versus zoledronic acid for treatment of bone metastases in men with castration-resistant prostate cancer: a randomised, double-blind study. *Lancet* 2011;377:813–22.
44. Ellis GK, Bone HG, Chlebowski R, Paul D, Spadafora S, Smith J, et al. Randomized trial of denosumab in patients receiving adjuvant aromatase inhibitors for nonmetastatic breast cancer. *J Clin Oncol* 2008;26:4875–82.

45. Gnant M, Pfeiler G, Dubsky PC, Hubalek M, Greil R, Jakesz R, et al. Adjuvant denosumab in breast cancer (ABCSG-18): a multicentre, randomised, double-blind, placebo-controlled trial. *Lancet* 2015;386:433–43.
46. Widschwendter M, Burnell M, Fraser L, Rosenthal AN, Philpott S, Reisel D, et al. Osteoprotegerin (OPG), the endogenous inhibitor of receptor activator of NF-kappaB Ligand (RANKL), is dysregulated in BRCA mutation carriers. *EBioMedicine* 2015;2:1331–9.
47. Schett G, Kiechl S, Redlich K, Oberhollenzer F, Weger S, Egger G, et al. Soluble RANKL and risk of nontraumatic fracture. *JAMA* 2004;291:1108–13.
48. Rogers A, Eastell R. Circulating osteoprotegerin and receptor activator for nuclear factor kappaB ligand: clinical utility in metabolic bone disease assessment. *J Clin Endocrinol Metab* 2005;90:6323–31.
49. Sigl V, Jones LP, Penninger JM. RANKL/RANK: from bone loss to the prevention of breast cancer. *Open Biol* 2016;6:1–9.