Research Article

Cancer Epidemiology, Biomarkers & Prevention

Reproductive and Lifestyle Factors and Circulating sRANKL and OPG Concentrations in Women: Results from the EPIC Cohort

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Abstract

Background: Except for a documented increase in osteoprotegerin (OPG) concentrations with older age, data on determinants of soluble Receptor Activator of Nuclear Factor κB (sRANKL) and OPG concentrations in women are limited. We evaluated reproductive and lifestyle factors as potential sources of variation in circulating sRANKL and OPG concentrations in pre- and postmenopausal women.

Methods: This study includes 2,016 controls [$n = 1{,}552$ (76%) postmenopausal, $n = 757$ (38%) using postmenopausal hormone therapy (PMH)] from a breast cancer case–control study nested in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. Serum sRANKL was measured using an ELISA and serum OPG using an electrochemiluminescent assay. Generalized linear models were used to evaluate associations between these analytes and reproductive and lifestyle factors.

Results: Older age at blood collection was associated with lower sRANKL concentrations in postmenopausal women ($P_{trend} \leq 0.03$) and higher OPG concentrations in all women ($P_{trend} \leq 0.01$). Longer duration of oral contraceptive use among premenopausal women and postmenopausal PMH users was associated with higher OPG ($P_{trend} \leq 0.04$). In postmenopausal non-PMH users, sRANKL concentrations were lower with longer duration of oral contraceptive use and current (vs. never) smoking ($P \leq 0.01$). sRANKL concentrations were higher among women with higher BMI ($P_{trend} \leq 0.01$). The evaluated factors accounted for 12% of the variation in sRANKL concentrations and 21% of the variation in OPG concentrations.

Conclusions: Circulating sRANKL and OPG concentrations are minimally impacted by hormone-related factors in pre- and postmenopausal women.

Impact: This study suggests circulating concentrations of sRANKL and OPG are unlikely to be strongly modified by hormone-related reproductive and lifestyle factors.

Introduction

The Receptor Activator of Nuclear Factor κB (RANK) axis includes the receptor, its ligand (RANKL) and the decoy receptor for RANKL, osteoprotegerin (OPG). Studies in experimental animal models have shown that the RANK axis plays an important role in a number of processes including bone turnover, immune response, cardiovascular disease, and breast development in pregnancy, as well as development and progression of breast cancer (1–3). With respect to breast cancer, we (4–6) and others (7–10) have observed associations

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between circulating concentrations of RANK axis members and breast cancer risk and prognosis. Following development of an antibody for RANKL, denosumab, there is increasing interest in RANKL and OPG in relation to breast cancer prevention and treatment.

Given the relevance of the RANK axis for a variety of outcomes, understanding whether concentrations of sRANKL (the soluble homotrimeric form of RANKL found in circulation) and OPG are potentially modulated by lifestyle and reproductive factors is of interest. Characterizing these associations contributes toward informing mechanistic understanding of associations between these factors and disease risk, providing an indication as to whether a subgroup of women may have particularly high concentrations due to lifestyle and/or reproductive-related factors, and elucidating whether circulating concentrations may be modifiable via changes in lifestyle and reproductive-related exposures. However, data to date on correlates of sRANKL and OPG concentrations in healthy individuals are limited. In a study of twins, genetic factors explained half of the variation in sRANKL concentrations, whereas OPG concentrations were almost entirely determined by environmental factors including age (11). A positive correlation between age and OPG concentrations has been reported by a number of studies (11–14), including our own (e.g., Spearman correlation = 0.29; ref. 5). We have also previously reported that OPG concentrations were higher among ever users of oral contraceptives (5). Given interest in circulating OPG and sRANKL for multiple disease outcomes, and following our prior observation of differences in OPG (5) and sRANKL (6) concentrations depending on postmenopausal hormone (PMH) use at blood collection, we conducted a detailed evaluation of associations between sRANKL and OPG concentrations and hormone-related reproductive and lifestyle factors in women by menopausal status and PMH use at blood collection.

Materials and Methods

Study population

The participants included in this study were women selected as controls in a case–control study on sRANKL and OPG concentrations and breast cancer risk ($n = 2{,}023$) nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. Data on reproductive, lifestyle, and anthropometric characteristics were collected for EPIC participants at baseline. Detailed descriptions of data collection with the EPIC cohort and design and baseline characteristics of the parent case–control study have been described in detail previously (5, 6, 15). For this study, eligible women were selected among cohort members who donated a baseline blood sample and were alive and free of cancer (except nonmelanoma skin cancer) at the time of diagnosis of their index case. Premenopausal women were eligible if they were not using exogenous hormones at baseline.

Laboratory assays

Serum concentrations of sRANKL and OPG were analyzed at the laboratory of the Division of Cancer Epidemiology at the German Cancer Research Center (DKFZ). Total OPG concentrations were measured using an electrochemiluminescence assay (MesoScale Diagnostics), a volume-efficient alternative to an ELISA. Free (i.e., non-OPG bound, or bioavailable) sRANKL concentrations were measured using an ELISA (Biomedica). The majority of sRANKL in circulation is bound to OPG and free sRANKL concentrations are known to be low (16); free sRANKL concentrations in our study were below the lower limit of detection of the assay (LLOD) in 152 women and were set to 50% of the LLOD (0.01 picomole per liter (pmol/L). Because of equipment failure two batches (38 women) of samples were not measured for sRANKL. A total of 4 women were missing sRANKL and/or OPG concentrations, leaving a total of 1,981 women with sRANKL and 2,019 women with OPG measured (Fig. 1). Interbatch coefficients of variation (CV) were 15.6% for premenopausal and 13.3% in postmenopausal women for sRANKL and 16.4% and 16.8%, respectively, for OPG. Intrabatch CVs were 0.9% and 1.5% for sRANKL in pre- and postmenopausal women, and 9.0% and 21.7% for OPG.

Statistical analyses

Outliers were evaluated using the extreme studentized deviate test (17); three women with outlying OPG concentrations (two with high concentrations and one with low concentrations) were identified and excluded (Fig. 1). No sRANKL outliers were identified. Means of natural log-transformed sRANKL and OPG concentrations were compared between categories of reproductive and lifestyle factors using adjusted generalized linear models. These results were exponentiated, providing the geometric mean in the original scale. Confidence limits were calculated subtracting/adding the SE multiplied by 1.96 (corresponding to the α for a two-sided P value of 0.05) from the geometric mean. P values for differences in concentrations across categories were calculated using type III sum of squares; P_{trend} was calculated by modeling the continuous variable as the exposure.

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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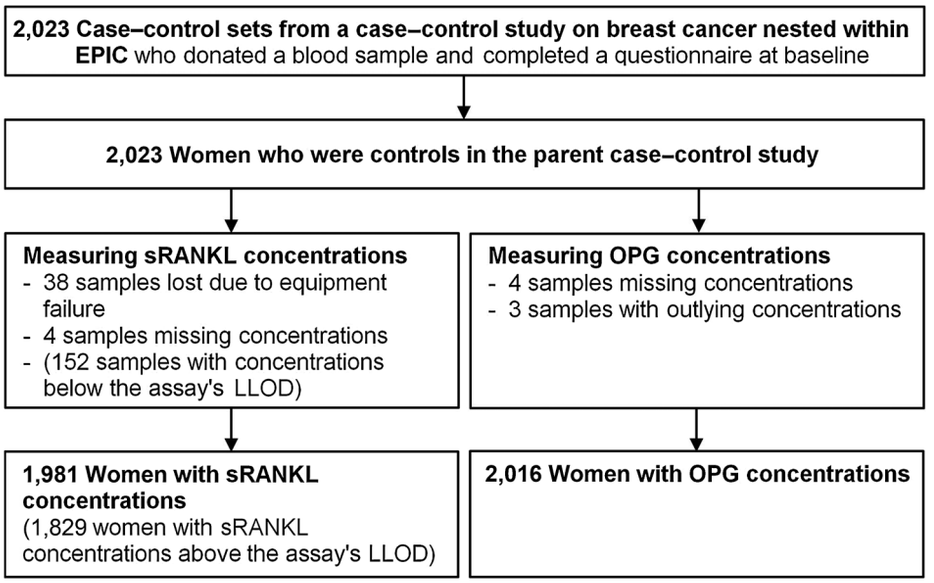

Figure 1.
Overview of participants from the parent case–control study who were eligible for the current study and in whom sRANKL and OPG concentrations were measured.

We evaluated age at blood collection (<50, 50–54, 55–59, 60+ years), age at menarche (<12, 12–14, 15+ years), oral contraceptive use (never, <5, 5–9, 10+ years), number of term pregnancies (none, 1, 2, 3+), and age at first-term pregnancy among women reporting ≥1 pregnancies (<25, 25+ years), body mass index (BMI; <20, 20–24.9, 25–29.9, 30+ kg/m^2), smoking status (never, former, current), and duration of smoking among ever smokers (≤10, 11–20, 21–30, 31–40, 41+ years), and total physical activity (inactive, moderately inactive, moderately active, active). The total physical activity variable includes household, recreational, and occupational activity, as described previously (18, 19). Additional variables of interest were ever breastfeeding and duration of breastfeeding among parous women reporting ever breastfeeding (<3, 3–6, 6–12, 12+ months), lifetime average alcohol consumption (never drinker, former drinker, >0–6, >6–12, >12 g/day), and fasting status at blood collection (<3, 3–6, 7+ hours). All analyses adjusted for the matching factors from the parent case–control study: country (as a proxy for study center), age (continuous), menstrual cycle phase (premenopausal women; early follicular, late follicular, ovulatory, early luteal, mid luteal, late luteal, missing; estimated using "backward dating" counting backward from start of menses following blood collection where data were available, otherwise estimated using "forward dating" as time since last menses (described in detail in ref. 20), fasting status (<3, 3–6, 7+ hours, missing), and time at blood collection (continuous), except when the variable was the exposure of interest. In a sensitivity analysis, oral contraceptive use and smoking status were mutually adjusted. Given our previous observation that sRANKL concentrations were lower (6), and OPG concentrations higher (5), among postmenopausal women using PMH, models were stratified by menopausal status and PMH use at blood collection. OPG and sRANKL concentrations did not vary by menstrual cycle phase in premenopausal women (5, 6). We estimated the proportion of variance explained by the epidemiologic factors using the linear regression model R^2 value.

Inclusion of participants with sRANKL values below the LLOD of the assay led to a nonnormal distribution of sRANKL concentrations; these subjects were excluded in a sensitivity analysis. All statistical tests were two-tailed and significant at $P < 0.05$. Statistical analyses were conducted using SAS 9.4 (SAS Institute Inc.).

Ethics approval and consent to participate

This project was approved by the International Agency for Research on Cancer (IARC) Ethics Committee (project no. 12-42) and the University of Heidelberg Ethics Commission (project no. S311/2014). The EPIC study protocol was approved by the ethical committees of IARC and the participating centers. All participants provided informed consent.

Results

Lower circulating sRANKL concentrations were observed with older age among women postmenopausal at blood collection ($P_{trend} \leq 0.03$; Table 1). There was no difference in concentrations by age among premenopausal women ($P_{trend} = 0.20$); however, the range in age was limited in this subgroup. In postmenopausal women not using PMH, both ever use of OCs and longer duration of OC use among ever users were associated with lower sRANKL concentrations ($P \leq 0.01$). Among postmenopausal PMH users who ever used oral contraceptives, longer duration of use was suggestively inversely associated with sRANKL concentrations ($P_{trend} = 0.07$). Parity ($P \geq 0.18$), and ages at menarche ($P_{cat} \geq 0.14$) and first full-term pregnancy ($P_{cat} \geq 0.19$) were not associated with sRANKL concentrations.

A higher BMI was associated with higher sRANKL concentrations regardless of menopausal status and PMH use at blood collection ($P_{trend} \leq 0.01$; Table 2). In postmenopausal women not using PMH at blood collection, there was suggestion of lower sRANKL concentrations in current smokers than in never smokers ($P_{cat} = 0.09$; Table 2); among women using PMH at blood collection current smokers had significantly

Table 1. Cross-sectional associations between reproductive characteristics and sRANKL concentrations (geometric means; pmol/L) by menopausal status and PMH use at blood collection

	Premenopausal women		Postmenopausal women			
			No PMH		PMH	
	n (%)[b]	Geometric mean (95% CI)	*n* (%)[b]	Geometric mean (95% CI)	*n* (%)[b]	Geometric mean (95% CI)
Full study population[a]	463 (100%)	0.07 (0.06–0.08)	760 (100%)	0.08 (0.07–0.10)	758 (100%)	0.06 (0.05–0.08)
Age at blood collection, years						
<50	370 (80%)	0.10 (0.08–1.13)	12 (2%)	0.09 (0.05–0.17)	31 (4%)	0.10 (0.06–0.17)
50–55	90 (19%)	0.08 (0.06–1.12)	123 (16%)	0.10 (0.07–0.12)	194 (26%)	0.08 (0.06–0.10)
50–60	3 (1%)	Not evaluated	279 (37%)	0.08 (0.06–0.09)	309 (41%)	0.07 (0.05–0.09)
60+			346 (46%)	0.07 (0.06–0.09)	224 (30%)	0.06 (0.04–0.08)
P_{trend}[c]		0.20		<0.01		0.03
Age at menarche, years						
<12	80 (17%)	0.11 (0.08–0.15)	101 (13%)	0.09 (0.07–0.12)	120 (16%)	0.06 (0.04–0.08)
12–14	321 (69%)	0.10 (0.08–0.12)	489 (64%)	0.08 (0.07–0.10)	484 (64%)	0.07 (0.06–0.09)
≥15	59 (13%)	0.08 (0.06–0.11)	159 (21%)	0.07 (0.05–0.09)	146 (19%)	0.07 (0.05–0.09)
P_{trend}[c]		0.16		0.14		0.93
Oral contraceptive use						
Never	165 (37%)	0.10 (0.08–0.13)	473 (65%)	0.08 (0.07–0.10)	282 (41%)	0.07 (0.05–0.09)
<5 years	157 (35%)	0.09 (0.07–0.12)	158 (18%)	0.09 (0.07–0.11)	164 (24%)	0.09 (0.06–0.12)
5–9 years	75 (17%)	0.10 (0.07–0.14)	50 (7%)	0.07 (0.05–0.10)	78 (11%)	0.06 (0.04–0.09)
≥10 years	49 (11%)	0.07 (0.05–0.10)	79 (11%)	0.06 (0.04–0.07)	169 (24%)	0.07 (0.05–0.09)
P_{trend}[d] including never users		0.08		<0.01		0.28
P_{trend}[c,e] among ever users		0.34		0.01		0.07
FTP						
Never	56 (12%)	0.10 (0.07–0.14)	95 (13%)	0.08 (0.06–0.10)	89 (12%)	0.06 (0.04–0.08)
Ever	401 (87%)	0.10 (0.08–0.13)	663 (87%)	0.08 (0.06–0.10)	647 (85%)	0.07 (0.05–0.09)
1 FTP	85 (21%)	0.10 (0.07–0.14)	109 (16%)	0.07 (0.05–0.09)	120 (19%)	0.06 (0.04–0.08)
2 FTPs	217 (54%)	0.10 (0.07–0.14)	308 (46%)	0.08 (0.06–0.10)	316 (49%)	0.07 (0.06–0.10)
≥3 FTPs	98 (24%)	0.09 (0.07–0.13)	246 (37%)	0.08 (0.06–0.10)	210 (32%)	0.07 (0.05–0.10)
P_{cat}[d] ever/never		0.77		0.76		0.21
P_{cat}[d,f] number		0.90		0.60		0.18
Age at first FTP[f]						
<25 years	192 (48%)	0.10 (0.07–0.13)	308 (46%)	0.08 (0.06–0.10)	369 (57%)	0.07 (0.05–0.09)
≥25 years	207 (52%)	0.10 (0.07–0.13)	353 (53%)	0.07 (0.06–0.09)	272 (42%)	0.07 (0.05–0.09)
P_{trend}[c]		0.87		0.30		0.19

NOTE: Generalized linear models adjusted for age (continuous), fasting status (<3, 3–6, >6 hours, missing), time of day (continuous), and menstrual cycle phase at blood collection (premenopausal women: early follicular, late follicular, ovulatory, early luteal, mid luteal, late luteal, missing), and country.
Abbreviations: CI, confidence interval; FTP, full-term pregnancy; PMH, postmenopausal hormone use.
[a]*P* value comparing pre- to postmenopausal at blood collection = 0.91; comparing PMH to no PMH use at blood collection <0.01.
[b]Percentages may not add up to 100% due to missing data.
[c]*P* value for linear trend assessed using type III F statistics from linear regression models with continuous exposure variables.
[d]*P* value for difference in geometric mean concentration of OPG across categories of the exposure variable. Assessed using type III F statistics from linear regression models.
[e]Among those who ever used oral contraceptives.
[f]Among those who ever had an FTP.

lower sRANKL concentrations relative to never smokers (P_{cat} = 0.01). The associations between oral contraceptive use and smoking and sRANKL concentrations were robust to mutual adjustment (data not shown). We found no association between alcohol consumption and sRANKL concentrations ($P \geq 0.22$).

Overall, the epidemiologic factors found to be associated with circulating sRANKL concentrations (plus the matching factors) explained 12% of the variability in this analyte in the full population; each individual variable explained <6% of the variation in sRANKL concentrations. Results were unchanged after excluding participants with sRANKL concentrations below the LLOD (Supplementary Tables S1 and S2).

OPG concentrations were highest among women with older age at blood collection ($P_{trend} \leq 0.01$; Table 3). Longer duration of oral contraceptive use was associated with higher OPG concentrations in premenopausal women (P_{trend} = 0.04) and postmenopausal women using PMH (P_{trend} < 0.01) in analyses including never oral contraceptive users. The association in premenopausal women was no longer statistically significant after adjustment for smoking (P_{trend} = 0.06; data not tabled), while the association was robust to this adjustment in postmenopausal women using PMH (P_{trend} < 0.01). No significant trend was observed in analyses restricted to ever users of oral contraceptives. Parity ($P_{cat} \geq 0.13$) and ages at menarche (P_{cat} = 0.16) and first full-term pregnancy ($P_{cat} \geq 0.11$) were not associated with OPG concentrations (Table 3); neither were any of the investigated lifestyle factors ($P \geq 0.09$; Table 4). Overall, the epidemiologic factors found to be associated with circulating OPG concentrations (plus the matching factors) explained 21% of the variability. The majority of this variability in OPG concentrations (18%) was explained by age.

In further analyses fasting status ($P_{trend} \geq 0.15$), breastfeeding history (P_{cat} = 0.17), and menstrual cycle phase ($P_{cat} \geq 0.18$) did not influence sRANKL or OPG concentrations (Supplementary Tables S3–S5). Among premenopausal parous women who breastfed, a longer duration of breastfeeding was associated with lower sRANKL concentrations ($P_{trend} \leq 0.03$; Supplementary Tables S3 and S4).

Table 2. Cross-sectional associations between lifestyle characteristics and sRANKL concentrations (geometric means; pmol/L) by menopausal status and PMH use at blood collection

	Premenopausal women		Postmenopausal women			
			No PMH		PMH	
	n (%)[a]	Geometric mean (95% CI)	*n* (%)[a]	Geometric mean (95% CI)	*n* (%)[a]	Geometric mean (95% CI)
Baseline BMI						
<20 kg/m^2	29 (6%)	0.07 (0.05–0.11)	48 (6%)	0.06 (0.04–0.09)	64 (8%)	0.06 (0.04–0.09)
20–24.9 kg/m^2	252 (54%)	0.09 (0.07–0.11)	322 (42%)	0.07 (0.06–0.09)	426 (56%)	0.06 (0.04–0.08)
25–29.9 kg/m^2	137 (30%)	0.11 (0.08–0.14)	285 (38%)	0.08 (0.07–0.11)	215 (28%)	0.09 (0.07–0.12)
≥30 kg/m^2	45 (10%)	0.13 (0.09–0.19)	105 (14%)	0.09 (0.07–0.12)	53 (7%)	0.10 (0.07–0.15)
P_{trend}[b]		<0.01		0.01		<0.01
Baseline smoking status						
Never smoker	245 (53%)	0.10 (0.08–0.13)	435 (57%)	0.08 (0.07–0.10)	395 (52%)	0.08 (0.06–0.10)
Former smoker	110 (52%)	0.10 (0.07–0.13)	178 (56%)	0.08 (0.06–0.10)	199 (59%)	0.06 (0.05–0.08)
Current smoker	103 (48%)	0.08 (0.06–0.11)	140 (44%)	0.07 (0.05–0.09)	138 (41%)	0.05 (0.04–0.08)
≤10 years[c]	45 (21%)	0.12 (0.08–0.19)	41 (13%)	0.08 (0.05–0.13)	55 (16%)	0.05 (0.03–0.08)
11–20 years[c]	62 (29%)	0.12 (0.08–0.18)	44 (14%)	0.05 (0.03–0.07)	50 (15%)	0.06 (0.03–0.11)
21–30 years[c]	87 (41%)	0.10 (0.07–0.15)	67 (21%)	0.08 (0.05–0.11)	73 (22%)	0.08 (0.05–0.13)
31–40 years[c]	13 (6%)	0.07 (0.04–0.14)	90 (28%)	0.07 (0.05–0.11)	95 (28%)	0.04 (0.03–0.07)
>40 years[c]	0	Not evaluated	58 (18%)	0.06 (0.04–0.09)	41 (12%)	0.05 (0.03–0.10)
P_{cat}[d] never vs. current smoking		0.15		0.09		0.01
P_{trend}[b,c] duration of smoking		0.15		0.65		0.58
Baseline physical activity						
Inactive	75 (16%)	0.09 (0.07–0.12)	80 (11%)	0.07 (0.05–0.10)	148 (20%)	0.07 (0.05–0.10)
Moderately inactive	145 (31%)	0.11 (0.08–0.14)	241 (32%)	0.07 (0.06–0.09)	319 (42%)	0.07 (0.06–0.10)
Moderately active	193 (42%)	0.09 (0.06–0.11)	381 (50%)	0.08 (0.07–0.10)	231 (30%)	0.07 (0.05–0.09)
Active	50 (11%)	0.09 (0.06–0.12)	58 (8%)	0.07 (0.05–0.10)	59 (8%)	0.07 (0.04–0.10)
P_{cat}[c] inactive vs. active		0.95		0.89		0.62
Lifetime alcohol consumption						
Nondrinker	40 (9)%	0.10 (0.07–0.16)	79 (10%)	0.08 (0.06–0.11)	27 (4%)	0.06 (0.03–0.10)
Former drinker	15 (3%)	0.08 (0.04–0.14)	41 (5%)	0.05 (0.04–0.08)	12 (2%)	0.07 (0.03–0.14)
Current drinker	395 (87%)	0.10 (0.08–0.14)	639 (81%)	0.08 (0.06–0.10)	696 (94%)	0.07 (0.05–0.09)
<6 gram/day[e]	144 (32%)	0.10 (0.07–0.14)	216 (28%)	0.08 (0.06–0.10)	181 (24%)	0.07 (0.05–0.09)
6–12 gram/day[e]	168 (37%)	0.11 (0.08–0.15)	295 (39%)	0.08 (0.06–0.11)	337 (45%)	0.07 (0.05–0.10)
≥12 gram day[e]	83 (18%)	0.09 (0.06–0.13)	110 (14%)	0.08 (0.06–0.10)	179 (24%)	0.06 (0.05–0.09)
P_{cat}[d] never vs. current alcohol use		0.94		0.86		0.46
P_{trend}[b] gram/day[e]		0.81		0.87		0.53

NOTE: Generalized linear models adjusted for age (continuous), fasting status (<3, 3–6, >6 hours, missing), time of day (continuous), and menstrual cycle phase at blood collection (premenopausal women: early follicular, late follicular, ovulatory, early luteal, mid-luteal, late luteal, missing), and country.
Abbreviations: BMI, body mass index; CI, confidence interval; PMH, postmenopausal hormone use.
[a]Percentages may not add up to 100% due to missing data.
[b]*P* value for linear trend assessed using type III F statistics from linear regression models with continuous exposure variables.
[c]Among current and former smokers.
[d]*P* value for difference in geometric mean concentration of sRANKL across categories of the exposure variable. Assessed using type III F statistics from linear regression models.
[e]Among current drinkers.

Discussion

We provide a large-scale cross-sectional study on reproductive and lifestyle factors in relation to sRANKL and OPG concentrations in healthy women. Our study suggests that circulating concentrations of these biomarkers are not strongly impacted by these characteristics. This study extends our prior work, where we reported Spearman correlations between sRANKL and OPG concentrations and circulating sex hormones, and age and BMI (5, 6); here we evaluated geometric mean concentrations for an extensive selection of epidemiologic characteristics, and assessed the total variance explained by these characteristics, to inform future studies incorporating these analytes.

RANKL is highly expressed in bone, lung, and lymph nodes and is found at lower levels in tissues including the placenta and heart (21). Circulating sRANKL is cleaved from RANKL expressed in tissue by metalloproteinases or formed by alternative messenger ribonucleic acid (mRNA) splicing (3). The RANK axis is an essential mediator of progesterone-induced proliferation in the breast during pregnancy in preparation for the formation of a lactating gland (22, 23). In addition to progesterone, prolactin and parathyroid hormone-related peptide, but not estradiol, have been shown to induce RANKL expression in mouse mammary tissue (24–28). While the main sources of circulating OPG appear to be bone marrow and vascular endothelial cells (29, 30), it is also produced by osteoblasts and production of OPG in the latter may be upregulated by estrogen and vitamin D, but not progesterone (13, 30–34).

OPG concentrations are known to increase with age (12, 13, 35), which was confirmed in our study. In line with our findings, weak inverse associations between age and sRANKL have been reported (6, 36, 37), with Spearman correlations < |0.13| observed in the current study population (6); however, the associations observed between age and sRANKL concentrations in prior studies are not entirely consistent and weak

Table 3. Cross-sectional associations between reproductive characteristics and OPG concentrations (geometric means; pmol/L) by menopausal status and PMH use at blood collection

	Premenopausal women		Postmenopausal women			
			No PMH		PMH	
	n (%)[b]	Geometric mean (95% CI)	*n* (%)[b]	Geometric mean (95% CI)	*n* (%)[b]	Geometric mean (95% CI)
Full study population[a]	464 (100%)	9.48 (9.08–9.91)	795 (100%)	9.80 (9.48–10.14)	757 (100%)	10.25 (9.88–10.63)
Age at blood collection, years						
<50	370 (80)	8.11 (7.64–8.60)	13 (2%)	8.55 (7.36–9.92)	31 (4%)	8.60 (7.80–9.49)
50–54	91 (19)	9.38 (7.75–9.10)	127 (16%)	8.80 (8.29–9.35)	194 (26%)	9.36 (8.86–9.88)
55–59	3 (1)	Not applicable	292 (37%)	9.70 (9.18–10.24)	308 (41%)	9.31 (8.82–9.82)
60+			363 (46%)	10.84 (10.30–11.40)	224 (30%)	10.60 (10.01–11.23)
P_{trend}[c]		0.01		<0.01		<0.01
Age at menarche						
<12 years	80 (17%)	7.84 (7.28–8.45)	104 (13%)	10.21 (9.54–10.93)	120 (16%)	9.49 (8.93–10.10)
12–14 years	322 (70%)	8.14 (7.68–8.63)	517 (66%)	9.89 (9.42–10.37)	483 (64%)	9.76 (9.27–10.27)
≥15 years	59 (13%)	8.22 (7.59–8.90)	163 (21%)	10.11 (9.53–10.71)	146 (19%)	9.78 (9.19–10.41)
P_{trend}[c]		0.18		0.23		0.16
Oral contraceptive use						
Never	166 (37%)	7.88 (7.39–8.40)	495 (65%)	9.92 (9.45–10.41)	282 (41%)	9.34 (8.83–9.89)
<5 years	157 (35%)	8.18 (7.68–8.72)	134 (18%)	10.13 (9.52–10.78)	164 (24%)	9.56 (8.99–10.16)
5–9 years	75 (17%)	8.19 (7.59–8.85)	51 (7%)	9.60 (8.81–10.47)	78 (11%)	9.70 (9.01–10.45)
≥10 years	47 (11%)	8.46 (7.77–9.22)	81 (11%)	10.23 (9.50–11.01)	168 (24%)	10.15 (9.53–10.81)
P_{trend}[d] including never users		0.04		0.64		<0.01
P_{trend}[c,e] among ever users		0.38		0.86		0.07
FTP						
Never	57 (12%)	7.98 (7.33–8.69)	102 (13%)	10.34 (9.69–11.04)	88 (12%)	9.53 (8.90–10.20)
Ever	401 (86%)	8.15 (7.66–8.67)	691 (87%)	9.92 (9.47–10.39)	647 (85%)	9.72 (9.26–10.21)
1 FTP	85 (21%)	8.24 (7.57–8.97)	110 (16%)	9.53 (8.91–10.21)	120 (19%)	9.78 (9.17–10.43)
2 FTPs	216 (54%)	8.13 (7.55–8.75)	317 (46%)	10.03 (9.51–10.58)	316 (49%)	9.64 (9.15–10.17)
≥3 FTPs	99 (25%)	8.33 (7.71–8.99)	264 (38%)	9.88 (9.35–10.44)	210 (32%)	9.68 (9.14–10.25)
P_{cat}[d] ever/never FTP		0.54		0.13		0.46
P_{cat}[d,f] number of FTP		0.66		0.22		0.86
Age at first FTP[f]						
<25 years	191 (48%)	8.30 (7.72–8.92)	318 (46%)	9.83 (9.33–10.36)	369 (57%)	9.74 (9.24–10.27)
≥25 years	208 (52%)	8.03 (7.46–8.64)	371 (54%)	9.98 (9.45–10.54)	272 (42%)	9.85 (9.32–10.40)
P_{trend}[c]		0.11		0.26		0.65

NOTE: Generalized linear models adjusted for age (continuous), fasting status (<3, 3–6, >6 hours, missing), time of day (continuous), and menstrual cycle phase at blood collection (premenopausal women: early follicular, late follicular, ovulatory, early luteal, mid luteal, late luteal, missing), and country.

Abbreviations: CI, confidence interval; FTP, full-term pregnancy; PMH, postmenopausal hormone use.

[a]*P* value comparing pre- to postmenopausal at blood collection = 0.57; comparing PMH to no PMH use at blood collection <0.01.

[b]Percentages may not add up to 100% due to missing data.

[c]*P* value for linear trend assessed using type III F statistics from linear regression models with continuous exposure variables.

[d]*P* value for difference in geometric mean concentration of OPG across categories of the exposure variable. Assessed using type III F statistics from linear regression models.

[e]Among those who ever used oral contraceptives.

[f]Among those who ever had an FTP.

positive (38, 39) and null findings (36) have also been reported. Longer duration of oral contraceptive use was associated with lower concentrations of sRANKL, and higher concentrations of OPG; the associations for sRANKL and for OPG among postmenopausal women not using PMH were robust to adjustment for smoking, while the OPG associations for premenopausal women were attenuated ($P = 0.06$ after adjustment; $P = 0.04$ before adjustment). Prior studies have reported positive associations between current oral contraceptive use and OPG concentrations, unadjusted for smoking, but no association for sRANKL (40, 41). To our knowledge, there are no prior studies on duration of oral contraceptive use and circulating sRANKL or OPG. It is plausible prolonged exposure to estrogens in oral contraceptives could increase OPG concentrations (and in turn lower free sRANKL concentrations), yet a mechanistic study did not observe changes in serum sRANKL or OPG in macaque monkeys after estrogen and progesterone treatment (42). Among the relatively small subset of parous women who ever breastfed ($n = 332$), longer duration of breastfeeding was associated with lower sRANKL concentrations. While the RANK–RANKL signaling is essential for breast development in pregnancy, we are not aware of any studies describing the role of the RANK axis in prolonged breastfeeding.

We observed higher concentrations of sRANKL with higher BMI, regardless of menopausal status at blood collection. While Uemura and colleagues observed no association between sRANKL and BMI in a population of postmenopausal women (39), others have reported a positive association with BMI in populations of men and women (43) and adolescents (44), as well as positive correlations between sRANKL and obesity-related markers of inflammation and insulin resistance (43). In line with previous studies (12, 39, 45, 46), OPG concentrations were similar across BMI categories in this study. Our finding of an inverse association between current smoking and sRANKL concentrations among postmenopausal women using PMH, and duration of smoking and sRANKL concentrations among postmenopausal women not

Table 4. Cross-sectional associations between lifestyle characteristics and OPG concentrations (geometric means; pmol/L) by menopausal status and PMH use at blood collection

	Premenopausal women		Postmenopausal women			
			No PMH		PMH	
	n (%)[a]	Geometric mean (95% CI)	*n* (%)[a]	Geometric mean (95% CI)	*n* (%)[a]	Geometric mean (95% CI)
Baseline BMI						
<20 kg/m^2	29 (6%)	7.76 (7.01–8.59)	48 (6%)	10.18 (9.35–11.09)	64 (8%)	10.31 (9.55–11.12)
20–24.9 kg/m^2	252 (54%)	8.17 (7.69–8.67)	336 (42%)	9.98 (9.49–10.51)	426 (56%)	9.72 (9.23–10.23)
25–29.9 kg/m^2	138 (30%)	8.10 (7.59–8.66)	299 (38%)	9.82 (9.32–10.35)	214 (28%)	9.612 (9.09–10.17)
≥30 kg/m^2	45 (10%)	8.09 (7.41–8.83)	112 (14%)	10.16 (9.53–10.82)	53 (7%)	9.41 (8.69–10.19)
P_{trend}[b]		0.74		0.60		0.23
Baseline smoking status						
Never smoker	247 (53%)	8.00 (7.53–8.50)	463 (58%)	9.94 (9.47–10.44)	395 (52%)	9.70 (9.21–10.22)
Former smoker	110 (52%)	8.18 (7.66–8.75)	181 (56%)	9.88 (9.33–10.45)	198 (59%)	9.62 (9.09–10.17)
Current smoker	102 (48%)	8.40 (7.83–9.00)	144 (44%)	10.27 (9.65–10.93)	138 (41%)	9.95 (9.35–10.59)
≤10 years[c]	45 (21%)	7.73 (7.04–8.49)	42 (13%)	9.88 (8.75–11.15)	55 (16%)	9.91 (8.93–11.01)
11–20 years[c]	62 (29%)	7.86 (7.18–8.60)	44 (14%)	10.44 (9.24–11.78)	50 (15%)	9.23 (8.25–10.31)
21–30 years[c]	86 (41%)	8.41 (7.73–9.16)	68 (21%)	10.03 (9.04–11.12)	73 (22%)	9.45 (8.54–10.46)
31–40 years[c]	13 (6%)	7.41 (6.41–8.57)	92 (28%)	10.97 (9.88–12.19)	94 (28%)	10.07 (9.11–11.14)
>40 years[c]	0	Not applicable	60 (18%)	9.90 (8.84–11.08)	41 (12%)	10.43 (9.22–11.81)
P_{cat}[d] never vs. current smoking		0.09		0.20		0.33
P_{trend}[b,c] duration of smoking		0.36		0.59		0.20
Baseline physical activity						
Inactive	75 (16%)	8.08 (7.49–8.72)	82 (10%)	9.82 (9.12–10.57)	148 (20%)	9.47 (8.89–10.10)
Moderately inactive	145 (31%)	8.03 (7.54–8.56)	247 (31%)	9.88 (9.34–10.44)	319 (42%)	9.75 (9.21–10.31)
Moderately active	193 (42%)	8.39 (7.86–8.94)	405 (51%)	10.00 (9.53–10.50)	230 (30%)	9.77 (9.26–10.30)
Active	51 (11%)	8.86 (7.26–8.52)	61 (8%)	10.25 (9.48–11.09)	59 (8%)	9.57 (8.87–10.34)
P_{cat}[d] inactive vs. active		0.52		0.33		0.78
Lifetime alcohol consumption						
Nondrinker	41 (9%)	8.04 (7.28–8.87)	88 (11%)	10.64 (9.87–11.47)	27 (4%)	10.40 (9.35–11.57)
Former drinker	15 (3%)	7.99 (6.95–9.18)	49 (6%)	11.13 (10.16–12.19)	12 (2%)	10.01 (8.67–11.55)
Current drinker	395 (85%)	8.11 (7.55–8.70)	639 (80%)	10.25 (9.69–10.84)	696 (93%)	9.87 (9.36–10.40)
<6 gram/day[e]	144 (31%)	8.23 (7.60–8.90)	219 (28%)	10.13 (9.52–10.78)	181 (24%)	10.01 (9.42–10.64)
6–12 gram/day[e]	168 (36%)	8.00 (7.42–8.62)	305 (38%)	10.36 (9.75–11.02)	337 (45%)	9.74 (9.21–10.30)
≥12 gram day[e]	83 (18%)	8.18 (7.53–8.89)	115 (14%)	1027 (9.57–11.02)	178 (24%)	9.95 (9.36–10.57)
P_{cat}[d] never vs. current alcohol use		0.83		0.22		0.27
P_{trend}[b] gram/day[e]		0.88		0.41		0.66

NOTE: Generalized linear models adjusted for age (continuous), fasting status (<3, 3–6, >6 hours, missing), time of day (continuous), and menstrual cycle phase at blood collection (premenopausal women: early follicular, late follicular, ovulatory, early luteal, mid luteal, late luteal, missing), and country.
Abbreviations: BMI, body mass index; CI, confidence interval; PMH, postmenopausal hormone use.
[a]Percentages may not add up to 100% due to missing data.
[b]*P* value for linear trend assessed using type III F statistics from linear regression models with continuous exposure variables.
[c]Among current and former smokers.
[d]*P* value for difference in geometric mean concentration of OPG across categories of the exposure variable. Assessed using type III F statistics from linear regression models.
[e]Among current drinkers.

using PMH is in agreement with prior studies in women (40) and a population of men and women (47).

No associations were observed between any of the investigated reproductive factors and sRANKL or OPG concentrations. For sRANKL, in particular, this may be reflective of weak associations between concentrations in the breast and circulating concentrations, as demonstrated by a previous study in macaques (42). In addition, we have shown that although concentrations of OPG were reproducible over time (Spearman *r* for serum samples taken fourteen years apart: 0.75), long-term reproducibility of sRANKL was lower (Spearman *r*: 0.38). This indicates one measurement of sRANKL may not reflect longer-term exposure (5, 6). While we observed no associations between hormone-related factors and circulating sRANKL and/or OPG among women predominantly older than age 50 in this study, it is plausible that these reproductive factors may impact concentrations in the shorter term, but may not be evident in the longer term, following these reproductive events.

At the time we initiated our study, there were no previous studies on sRANKL or OPG and breast cancer, which was the focus of the parent study. Three studies published since used ELISA assays to measure OPG concentrations (7, 8, 42). The two studies that report OPG concentrations observed different ranges of OPG concentrations in their study populations: 0.46–25.81 ng/mL (23.12–1,296.98 pmol/L; ref. 7) and 4.2–547.7 ng/mL (211.06–27,522.61 pmol/L; ref. 8); however, the two studies used different ELISAs. Both of these reported concentrations are higher than the range of concentrations, we observed in the current study (3.54–33.02 pmol/L). Given the lack of a cross-assay standardization protocol, comparison between studies relies on relative within-study differences in concentrations. The ELISA we used to quantify free sRANKL concentrations in our study is the most sensitive assay currently commercially available, but median free sRANKL concentrations observed in our study were relatively low (0.11 pmol/L) and 7.5% ($n = 152$) of the study population had concentrations below the

LLOD of the assay. One previous study used the same assay to measure sRANKL, but did not report mean or median concentrations, or if any samples were measured to be below the LLOD (9). Total serum sRANKL concentrations may be over 1,000 pmol/L, but the majority of sRANKL found in circulation is bound to OPG (16) and cannot bind to RANK to activate signaling. Thus, even though free sRANKL concentrations are low, they are likely a more informative measure than total sRANKL concentrations.

A further limitation to our study is the relatively high interbatch CVs, especially for OPG concentrations in postmenopausal women, indicating potential measurement error that may have attenuated findings. In addition, one small previous study suggests sRANKL and OPG concentrations may decrease over time in serum samples stored at temperatures higher than −70°C (48). Serum samples in our study were stored at −150°C and we observed no correlation between sRANKL and OPG concentrations and date of blood collection (Spearman $r \leq 0.03$; samples collected between 1992 and 2002). R^2 values indicate that the evaluated factors explain relatively little of the variation in sRANKL and OPG concentrations. However, this may be influenced by the limited variability in the distributions of the examined factors in our population of middle to older aged European women. Finally, due to multiple comparisons, some of our results may be due to chance.

In this large cross-sectional study, we demonstrate that circulating sRANKL and OPG concentrations are minimally impacted by hormone-related factors in women. In the context of the limited evidence to date, these results suggest circulating concentrations of sRANKL and OPG are unlikely to be modifiable by lifestyle or reproductive factors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The authors alone are responsible for the views expressed in this article and they do not necessarily represent the views, decisions, or policies of the institutions with which they are affiliated.

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Availability of Data and Material

For information on how to submit an application for gaining access to EPIC data and/or biospecimens, please follow the instructions at http://epic.iarc.fr/access/index.php.

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