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Premenopausal plasma osteoprotegerin and breast cancer risk: a case-control analysis nested within the Nurses' Health Study II

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Abstract

Background: Emerging evidence supports a role of the receptor activator of nuclear factor κB (RANK) pathway in normal mammary gland development and breast carcinogenesis. Osteoprotegerin (OPG) is the endogenous decoy receptor for RANK-ligand (RANKL) that inhibits RANK-signaling. Whether OPG may be a biomarker of breast cancer risk remains unclear.

Methods: We evaluated the association between plasma OPG and breast cancer risk in a case (n=297)-control (n=297) study nested within the Nurses' Health Study II. Cases were women who were cancer-free and premenopausal at blood collection who developed invasive breast cancer. OPG was quantified using an enzyme-linked immunosorbent assay. Conditional logistic regression was used to estimate multivariable odds ratios (ORs) and 95% confidence intervals (CI) for the association between OPG levels and breast cancer risk adjusting for potential confounders. Unconditional logistic regression, additionally adjusting for matching factors, was used for stratified analyses.

Results: Overall, there was no substantial evidence for an association between plasma OPG levels and breast cancer risk, though the point estimate for the highest (vs. lowest) quartile was below one (OR=0.78; 95%CI 0.46-1.33; *P*-trend=0.30). There was no evidence of heterogeneity

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by various reproductive, hormonal, or tumor characteristics including hormone receptor status and grade (all *P*-heterogeneity ≥0.17).

Conclusions: Findings from this prospective study do not provide substantial evidence for an association between circulating OPG and breast cancer risk among premenopausal women; however, we were underpowered in stratified analyses.

Impact: Results do not provide strong evidence for OPG as a potential biomarker of breast cancer risk among premenopausal women.

INTRODUCTION

There is a growing body of experimental evidence demonstrating an important role of the receptor activator of nuclear factor κB (RANK)/RANK ligand (RANKL) pathway in both normal mammary gland development and breast carcinogenesis [1-5]. Binding of RANKL to its cognate receptor RANK promotes proliferation, differentiation, and migration of mammary epithelial cells[1,3], while osteoprotegerin (OPG) is the endogenous decoy receptor for RANKL that inhibits the action of RANKL and thus antagonizes RANK-signaling[6]. As a result, it is of interest to identify additional markers that may improve current understanding of the etiologic role of the RANK pathway in breast cancer development, improve upon risk prediction, and potentially offer insight into novel targets for prevention.

Emerging reports of aberrant circulating levels of RANKL and OPG among women with an inherited *BRCA1* or *BRCA2* mutation suggest that dysregulation of this pathway may be implicated in breast cancer predisposition [7,8]. Thus, OPG levels may have the potential to help stratify women who are at risk of developing disease, and furthermore, may provide insight into the pathogenesis of breast cancer and novel targets for prevention. However, the relationship between OPG and breast cancer risk among women in the general population is unclear, given that findings from the relatively few epidemiologic studies conducted to date remain inconclusive[9-12]. In addition, most studies have been conducted among postmenopausal women and there have been varying methods utilized to quantify OPG.

Given the inhibitory role of OPG in the RANKL-signaling pathway, we hypothesized that premenopausal women with higher circulating levels of OPG would be at lower risk of breast cancer. Thus, we aimed to assess the association between circulating plasma OPG and breast cancer risk among premenopausal women in a case-control study nested within the Nurses' Health Study II. We also aimed to evaluate whether this association varied by menopausal status at diagnosis, as well as various hormonal and tumor characteristics.

MATERIALS AND METHODS

Study population

We performed a matched case-control study nested within the Nurses' Health Study II (NHSII). Details on the NHSII cohort have been described elsewhere [13]. Briefly, NHSII is a prospective cohort study that was initiated in 1989. A total of 116,429 female registered nurses aged 25-42 years residing in 14 states across the United States were recruited at baseline. Women have since been followed via biennial questionnaires. Between 1996 and

1999, 29,611 NHSII participants aged 32-54 years provided blood samples. Blood collection procedures have been described previously[13]. Briefly, blood samples were drawn and shipped on ice via overnight courier to the NHS central laboratory. Samples were aliquoted at the laboratory into plasma, white blood cell, and red blood cell components and have since been stored at ≤130°C in continuously monitored liquid nitrogen freezers. Follow-up of the blood cohort was 95% in 2009.

Ethical approval for this study was obtained from the institutional review boards of the Brigham and Women's Hospital, Harvard T.H. Chan School of Public Health, and participating registries, as required.

Case and control selection

Cases were women who were cancer-free and premenopausal at blood collection who developed incident invasive breast cancer after blood collection but before June 1, 2007. All breast cancer cases included in this analysis were self-reported and then confirmed via medical record review. Controls were selected via risk-set sampling and individually matched to cases in a 1:1 ratio on case diagnosis date, age at blood collection (+/− 2 years), time of blood collection (month [+/− 1 month] and time of day [+/− 2 hours]), fasting at blood collection (<2, 2-4, 5-7, 8-11, ≥12 hours since last meal), menopausal status in the questionnaire cycle before cancer diagnosis/control index date (premenopausal [self-reported continuing menstrual cycles], postmenopausal [no menstrual cycles in last 12 months, surgical menopause with bilateral oophorectomy or age ≥54 for smokers, ≥56 for non-smokers], unknown), and self-reported race/ethnicity (white, non-white). In addition, women who gave a blood sample timed in the menstrual cycle (n=470) were matched on luteal day of the menstrual cycle (defined as the date of a woman's next period minus the date of the luteal blood draw, ± 1 day). There were no premenopausal controls for seven of the cases, and thus, they were matched to seven controls with either an unknown menopausal status or who were postmenopausal.

Laboratory assays

We measured absolute plasma OPG levels in pmol/L from a single blood sample collected between 1996 and 1999. Plasma OPG was measured at Dr. Nader Rifai's laboratory (Boston Children's Hospital, Boston, MA) using an enzyme-linked immunosorbent assay (ELISA) that employs a quantitative sandwich enzyme immunoassay technique (Alpco Diagnostics, Salem, NH). The limit of detection of the assay was 0.17 pmol/L. A total of 300 case-control sets were tested for OPG. We excluded 3 case-control sets for whom either the case's (n=1) or the control's (n=2) OPG levels could not be measured as a result of inadequate plasma samples (cracked sample vial [n=2] or hemolysis [n=1]). Matched case-control sets were handled identically and were assayed together in the same batch. The ordering of each matched-case-control set was randomly assigned, and laboratory personnel were blind to case-control status. To evaluate assay reliability, blinded replicates from pooled samples (~10% of all samples) were interspersed among the samples and were used to calculate coefficients of variation (CV). The inter-batch CV for OPG from blinded replicate samples was 7.8%. The intra-batch CV was 2.6%.

Plasma concentrations of circulating luteal progesterone, average prolactin (follicular/luteal), follicular estradiol, luteal estradiol, total testosterone, and 25-hydroxyvitamin D were available on a subset of the study population (n=416-591) and were assessed as potential effect modifiers. All biomarkers were measured from the same blood sample that was used for OPG analyses. Descriptions of laboratory methods have been published previously [13-15].

Statistical analyses

Descriptive statistics were used to summarize the distributions of cases and controls by demographic and epidemiologic characteristics. The Wilcoxon signed-rank test for matched data was used to evaluate differences in OPG levels between cases and controls. Women were categorized into quartiles of OPG based on the levels in the controls, with the lowest quartile as the reference group. Odds ratios (ORs) and 95% confidence intervals (CIs) for the association between quartiles of OPG and breast cancer risk were estimated using conditional logistic regression [16]. Because potential confounders of the OPG-breast cancer relationship remain unclear, we assessed three different models: model A accounted for the matching factors listed above, model B accounted for matching factors and potential confounders for which there was a strong biologic rationale or that appeared to be associated with OPG levels in bivariate analyses, and model C accounted for matching factors and all known potential confounders identified *a priori* using directed acyclic graphs [17]. Potential confounders were identified from the questionnaire administered at the time of blood collection or the biennial questionnaire immediately preceding blood collection, unless otherwise noted, and included: age at menarche (continuous: years); parity/age at first birth (categorical: nulliparous, 1-2 pregnancies/age first birth<25, 1–2 pregnancies/age first birth ≥25, ≥3 pregnancies/age first birth <25, ≥3 pregnancies/age first birth≥25); ever breastfed (categorical: yes, no); family history of breast cancer (categorical: yes, no); history of biopsy-confirmed benign breast disease (categorical: yes, no); BMI at age 18 (continuous: kg/m^2); weight change between age 18 and blood collection (continuous: kg); average alcohol consumption from 1991 and 1995 questionnaires (continuous: grams/day); and average physical activity from 1989, 1991, and 1997 questionnaires (continuous: Metabolic Equivalent of Task [MET]-hrs/week). We examined linear trends by modeling the median of each OPG quartile as a continuous variable and testing for linearity using the Wald test.

We also assessed associations between OPG levels and categorical breast cancer risk factors using adjusted geometric means. We used partial Spearman correlation coefficients to evaluate associations between OPG levels and continuous breast cancer risk factors, circulating plasma hormone levels, plasma 25-hydroxyvitamin D, and percent mammographic density (obtained from a mammogram taken as close to the date of blood collection as possible, as described previously)[18]. These analyses were adjusted for matching factors and restricted to controls.

In addition, we used restricted cubic splines to assess the potential for a non-linear relationship between OPG and breast cancer risk non-parametrically [19]. Knots were placed at the 5^{th}, 27.5^{th}, 50^{th}, 72.5^{th}, and 95^{th} percentiles. Tests for non-linearity used the likelihood ratio test, comparing the model with only the linear term to the model with the linear and the cubic spline terms.

Sensitivity analyses

We performed several pre-specified sensitivity analyses based on prior literature and *a priori* hypotheses. Given the potential for heterogeneity of the observed associations by tumor characteristics, we evaluated OPG associations separately by tumor subtype (ER+ vs. ER-; PR+ vs. PR-; ER+/PR+ vs. ER-/PR- vs. ER+/PR-), tumor grade (1, 2, 3), and tumor size (<2 vs. ≥2 cm). We assessed potential heterogeneity using unconditional nominal polytomous logistic regression adjusted for matching factors and potential confounders, testing for heterogeneity using the Wald test and allowing the effects of covariates to vary by tumor characteristic [20]. We also conducted stratified analyses by several factors identified a *priori* as potential effect modifiers: menopausal status at the questionnaire cycle before cancer diagnosis/control index date (premenopausal vs. postmenopausal), luteal progesterone, average prolactin (follicular/luteal), follicular estradiol, luteal estradiol, total testosterone, and 25-hydroxyvitamin D levels (all dichotomized at the median among controls), percent mammographic density (dichotomized at the median among controls), BMI at blood collection (<25 vs. ≥25 kg/m^2), and fasting status at blood collection (<8 vs. ≥8 hours). Family history of breast cancer, which is another potentially important effect modifier, was not included due to the limited number of women with a positive family history. For stratified analyses, we used unconditional logistic regression, controlling for matching factors and confounders. Likelihood ratio tests were used to compare models with and without interaction terms between each stratification variable and OPG (quartiles) to evaluate potential effect-measure modification on the multiplicative scale.

To assess potential reverse causality and variations according to lag time, we conducted an analysis stratified by time from blood collection to breast cancer diagnosis/control index date (<2 years, 2–<5 years, 5–<8 years, ≥8 years). A likelihood ratio test was used to assess potential multiplicative effect-measure modification by lag time.

We corrected effect estimates and confidence intervals for random within-person variability and laboratory measurement error using intraclass correlation coefficient (ICC) calibration [21]. ICCs were calculated from a prior reproducibility study conducted within the first Nurses' Health Study (NHS), with plasma OPG measured for each woman at two time points approximately one year apart.

Statistical outliers for OPG were identified using the generalized extreme Studentized deviate many-outlier procedure [22]. In a sensitivity analysis, potential outliers identified using this approach were excluded from the analysis, and these results were compared to the main findings.

Finally, given percent mammographic density is an important risk factor for breast cancer [23] and the relationship between OPG and mammographic density remains unclear, in a subgroup of 416 participants with mammographic density data we assessed associations between plasma OPG (categorized into tertiles among controls) and percent mammographic density using multivariable linear regression adjusted for matching factors and potential confounders. Based on *a priori* hypotheses, we also assessed associations between OPG tertiles and mammographic density stratified by 25-hydroxyvitamin D, luteal progesterone,

average prolactin, follicular estradiol, luteal estradiol, total testosterone, and BMI at blood collection (as categorized previously).

All statistical tests were two-sided and analyses were conducted using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS

A total of 297 cases and 297 matched controls were included in this analysis. Cases and controls were similar with respect to most breast cancer risk factors except for the proportion of women who were nulliparous, had a history of benign breast disease, and had family history of breast cancer, which was slightly higher among cases than among controls (Table 1). Characteristics of the breast cancer cases included in the analysis are summarized in Supplemental Table 1. The majority of cases were premenopausal at the time of their diagnosis (73%), ER+/PR+ (69%), and HER2– (60%). The median age at diagnosis was 49.7 years (range 37.1-59.3). Most of the cancers were <2 cm in size (70%) and moderately (36%) or poorly (34%) differentiated. The median time to breast cancer diagnosis was 5.1 years (IQR 2.6-7.3).

There were no significant differences in the adjusted geometric mean values of plasma OPG by various categorical breast cancer risk factors among the controls (all *P*-values ≥ 0.08) (Supplemental Table 2). There was also no significant correlation between plasma OPG and various continuous breast cancer risk factors, circulating plasma hormone levels, plasma 25-hydroxyvitamin D, or percent mammographic density, except for weak positive correlations with age at blood collection (partial spearman correlation coefficient = 0.19; *P*-value = 0.001) and luteal progesterone (partial spearman correlation coefficient = 0.16; *P*-value = 0.01) (Supplemental Table 3).

There was no substantial difference in median pre-diagnostic OPG levels between cases and controls (3.54 pmol/L [IQR 3.02-4.11] among cases vs. 3.64 pmol/L [3.12-4.31] among controls; Wilcoxon signed-rank test *P*-value = 0.12). There was also no statistically significant association between quartiles of plasma OPG and overall breast cancer risk, though there was a suggestive inverse trend (Table 2). The OR and 95% CI comparing women in the highest quartile of OPG to those in the lowest quartile of OPG in the model only adjusting for the matching factors was 0.67 (95% CI 0.41-1.11; *P*-trend = 0.11). The corresponding risk estimates were 0.79 (95% CI 0.46-1.33; *P*-trend = 0.30) in model B and 0.78 (0.46-1.33 *P*-trend = 0.30) in model C which additionally adjusted for potential confounders. Using non-parametric restricted cubic splines, there was no evidence of a non-linear relationship between OPG and breast cancer risk (likelihood ratio test *P*-value for non-linearity = 0.78).

We also evaluated the relationship between OPG and breast cancer risk in analyses stratified by various epidemiologic risk factors, tumor characteristics, and sex hormone levels (Table 3). There was no significant heterogeneity in the association between circulating OPG and the risk of developing breast cancer by any of the reproductive, anthropometric, or hormonal characteristics that we evaluated (all *P*-interaction ≥ 0.17). However, although there was no

significant interaction, increasing plasma OPG was associated with lower risk of breast cancer among women with luteal estradiol below the median (*P*-trend = 0.02). The relationship between OPG and breast cancer risk did not vary by tumor hormone receptor status, grade, or size (all *P*-heterogeneity ≥ 0.61).

In an analysis stratified by time from blood collection to breast cancer diagnosis/control index, there was no statistical evidence of effect-measure modification by lag time; however, risk estimates tended towards more protective effects with increasing lag times. After correction for measurement error using OPG reproducibility data from NHSI (OPG ICC=0.72 [95% CI 0.62-0.81]), the results were not substantially altered, though corrected effect estimates tended to be slightly stronger (odds ratio [95% CI] comparing the median OPG level of women in the highest vs. lowest quartiles was 0.80 [0.51-1.27] before measurement error correction vs. 0.72 [0.35-1.44] after measurement error correction). Findings were similar when potential outliers (n=7) were excluded from the analysis. Finally, there was no substantial evidence of an association between plasma OPG and percent mammographic density (estimate for mean difference in percent mammographic density comparing the highest versus the lowest tertile of OPG=2.83; 95% CI −1.28-6.95; *P*-trend = 0.18). There was also no evidence of an association between OPG and mammographic density in stratified analyses.

DISCUSSION

Activation of the RANK/RANKL pathway promotes proliferation, differentiation, and migration of mammary epithelial cell [1,3] as well as expansion and survival of mammary stem cells [4]. Given the important role of the RANK-signaling pathway in both normal mammary gland development and in mammary carcinogenesis, we evaluated whether levels of OPG, the endogenous inhibitor of this pathway, were associated with breast cancer risk in premenopausal women. Using pre-diagnostic plasma OPG levels from 297 cases and 297 matched controls enrolled in the NHSII, we observed no substantial evidence of an association between circulating levels of OPG and breast cancer risk, although estimates tended towards a suggestive inverse association. We also evaluated whether the association between OPG and breast cancer risk varied by participant characteristics (i.e., menopausal status at diagnosis) or tumor markers (i.e., ER/PR status). There was no significant heterogeneity in our findings, although some results were based on small strata in the subgroup analyses. Thus, although our findings do not provide substantial evidence of an association between circulating OPG and breast cancer risk among premenopausal women, our analyses may have been underpowered.

To date, there have been four reports of circulating (serum) OPG and/or RANKL levels and subsequent risk of breast cancer among women in the general population. In a prospective study conducted in Norway which included 3,174 women but only 76 incident breast cancers, Vik *et al.* observed a borderline significant inverse relationship between serum OPG and breast cancer risk (HR tertile 3 vs. tertile 1 = 0.55; 95%CI 0.28-1.08; *P*-trend = 0.08). This association was stronger among women younger than 60, and there was no association observed among women over age 60.

In a recent nested case-control study within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (2,008 cases/2,008 controls), Fortner *et al.*, reported significant heterogeneity between serum OPG levels and risk of breast cancer by ER-status of the tumor[9]. Increasing OPG was associated with an increased risk of ER− (n = 368 cases) but not ER+ disease (n = 1,622). There was also evidence for heterogeneity by menopausal status at blood collection among women with ER+ tumors. The relative risk for each unit increase in log2-transformed OPG was 0.53 (95%CI 0.31-0.88) for premenopausal women while it was 0.97 (95%CI 0.75-1.25) for postmenopausal women. Our suggestive findings are in line with the inverse association reported among premenopausal women in the Fortner *et al.,* publication. In an additional publication from the same cohort, Sarink *et al.,* investigated the relationship between serum RANKL and RANKL/OPG ratio and breast cancer risk (n = 1,976 cases/1,976 controls)[11]. Women in the highest vs. lowest quartile of circulating RANKL had an increased risk of ER+ breast cancer but not ER— disease. Findings were similar for the RANKL/OPG ratio, and, the increased risk with both RANKL and RANKL/OPG ratio was limited to women with ER+ disease diagnosed after age 50.

In the final report which evaluated OPG, RANKL, and RANKL/OPG ratio among 278 postmenopausal women enrolled in the UK Collaborative Trial of Ovarian Cancer Screening study (UKCTOCS), Kiechl *et al.*, reported an approximately five-fold increased risk of breast cancer among women who had high progesterone and RANKL levels or high progesterone and a high RANKL/OPG ratio; however, this was only apparent among women who provided blood 12-24 months prior to diagnosis [10]. In addition, findings from this study were difficult to interpret given the small strata and large confidence intervals.

In the present study, we observed a suggestion towards an inverse association between OPG levels and breast cancer risk. We also conducted several analyses stratified by various reproductive and lifestyle factors, circulating hormone levels, and tumor characteristics. There was evidence of a potential inverse association between plasma OPG and breast cancer risk among women with low luteal estradiol; however, given the multiple comparisons and lack of known biologic rationale, this result may represent a chance finding. We also observed a positive correlation between plasma OPG and age which is consistent with previous reports [9,24,25]; however, the weak, positive correlation we observed between OPG and luteal progesterone requires additional study given that others have reported either no relationship or an inverse relationship, although the latter was only observed in women with a *BRCA1* or *BRCA2* mutation[7,9]. There were no other significant correlations with other risk factors or hormones which is in line with previous reports suggesting that circulating concentrations of OPG may not be influenced by hormonal or lifestyle factors [9,24,25].

Given our modest sample size and considering our findings are in line with two previous reports [9,12], the suggestive inverse association between circulating OPG levels and overall breast cancer risk among premenopausal women suggests that it is still plausible that this protein may serve as a clinical biomarker to stratify women at the highest risk of developing disease. However, this hypothesis requires confirmation in a larger sample of premenopausal women, as well as in high-risk populations such as those with inherited *BRCA1* or *BRCA2* mutation or a strong family history of disease[26]. Indeed, there is preclinical evidence

suggesting dysregulated RANK-signalling in the pathogenesis of *BRCA1*-associated breast cancer may be of relevance to other subsets of women[7,16,27]. In addition, given OPG may also influence breast cancer development through other pathways, for example by binding to TNF-related apoptosis-inducing ligand (TRAIL) and inhibiting cancer cell death or influencing angiogenesis and inflammation [28-30], future studies should consider including additional markers to more fully understand the potential mechanisms of OPG.

Strengths of the current study include the prospective study design, the inclusion of finely matched cases and controls and detailed information on relevant potential confounders and breast cancer risk factors. We also corrected effect estimates for intra-individual and laboratory measurement error using a previous reproducibility study. Our study also has several limitations. We assessed circulating OPG using a single measurement; however, previous research has demonstrated that this protein is highly reproducible over time including over a 14 year period (r=0.85) [9]. Furthermore, others have shown no substantial variation in OPG concentrations throughout the menstrual cycle using timed blood sample [7,9,24]. In addition, our relatively small sample size may have influenced our statistical power in analyses stratified by various reproductive, hormonal, or tumor characteristics. We did not measure RANKL concentrations, the cytokine ligand for RANK, which may also influence breast cancer development. Finally, although a circulating biomarker (i.e., plasma OPG) may not be reflective of the tissue of interest (i.e., breast or mammary tissue OPG), it has been previously demonstrated that progesterone-induced changes in breast OPG levels directly correlated with changes in circulating OPG, suggesting that blood OPG is a valid proxy of breast OPG [7].

Findings from our study nested within the Nurses' Health Study II cohort do not provide substantial evidence for a relationship between circulating levels of OPG and subsequent breast cancer risk, although a suggestive inverse trend was observed and our study was limited by the relatively modest number of women included. It is of interest to further evaluate whether there is a role of OPG as a marker of risk using larger populations across a broad range of underlying risk, defined by *BRCA* mutation status, polygenic risk score, and other risk prediction models[6]. Thus, given the collective evidence suggestive of a potential inverse relationship between OPG and risk in younger women, further investigation using large cohorts of premenopausal women is warranted and may provide insight into novel targets for chemoprevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Characteristics of breast cancer cases and matched controls in a nested case-control study within the Nurses' Health Study II.[1]

Characteristic	Cases (*n*=297)	Controls (*n*=297)
Age at blood collection (years), median (IQR)	44.2 (41.3 – 47.0)	44.1 (41.5 – 46.8)
Age at menarche (years), median (IQR)	12.0 (12.0 – 13.0)	12.0 (12.0 – 13.0)
Race/ethnicity, white, n (%)	293 (98.7%)	294 (99.0%)
BMI at age 18 (kg/m^2), median (IQR)	20.2 (18.9 – 22.0)	20.5 (19.2 – 22.1)
BMI at blood collection (kg/m^2), median (IQR)	23.6 (21.6 – 27.2)	23.8 (21.2 – 28.3)
Weight change from age 18 to blood collection (kg), median (IQR)	9.1 (3.6 – 16.4)	9.1 (3.6 – 18.2)
Fasting at blood collection (≥8 hours since last meal), n (%)	191 (64.3%)	198 (66.7%)
Premenopausal at diagnosis/index date, n (%)	218 (73.4%)	214 (72.1%)
Nulliparous, n (%)	66 (22.2%)	52 (17.5%)
Parity, median (IQR)[2]	2.0 (2.0 – 3.0)	2.0 (2.0 – 3.0)
Age at first birth (years), median (IQR)[2]	27.0 (24.0 – 29.0)	26.0 (23.0 – 29.0)
Ever breastfed, n (%)	188 (63.3%)	196 (66.0%)
History of biopsy-confirmed benign breast disease, n (%)	59 (19.9%)	46 (15.5%)
Family history of breast cancer, n (%)	49 (16.5%)	33 (11.1%)
Average alcohol consumption (grams/day), median (IQR)[3]	1.2 (0 – 4.1)	1.2 (0 – 3.8)
Average physical activity (MET-hrs/week), median (IQR)[4]	14.9 (7.7 – 29.2)	16.6 (8.5 – 31.7)
Osteoprotegerin (pmol/L), median (IQR)	3.54 (3.02 - 4.11)	3.64 (3.12 - 4.31)

[1]Cases and controls were matched on case diagnosis date, age at blood collection (+/− 2 years), time of blood collection (month [+/− 1 month] and time of day [+/− 2 hours]), fasting at blood collection (<2, 2-4, 5-7, 8-11, ≥12 hours since last meal), menopausal status at blood collection and in the questionnaire cycle before cancer diagnosis/control index date (premenopausal, postmenopausal, unknown), and self-reported race/ethnicity (white, non-white). In addition, women who gave a blood sample timed in the menstrual cycle (*n*=470) were matched on luteal day.

[2]Parity and age at first birth among parous women.

[3]Average alcohol consumption from the 1991 and 1995 questionnaires.

[4]Average physical activity from the 1989, 1991, and 1997 questionnaires.

NOTE: Missing values for age at menarche (*n*=3 [0.5%]), BMI at age 18 (*n*=1 [0.2%]), weight change between age 18 and blood collection (*n*=1 [0.2%]), age at first birth (*n*=2 [0.3%]), and alcohol consumption (*n*=15 [2.5%]) were imputed using the median (continuous covariates) or the mode (categorical covariates).

Abbreviations: IQR=interquartile range; BMI=body mass index; QX=questionnaire; MET=Metabolic Equivalent of Task.

Table 2.

Associations between pre-diagnostic plasma osteoprotegerin quartiles and invasive breast cancer in a matched case-control study nested within the Nurses' Health Study II.

Osteoprotegerin (pmol/L)	No. of Cases	No. of Controls	Odds Ratio (95% CI)	*P*-value [1]
Model A[2]				
Quartile 1 (0.4-3.1)	85	75	Reference	--
Quartile 2 (3.1-3.6)	80	74	0.93 (0.61, 1.44)	0.76
Quartile 3 (3.7-4.3)	72	74	0.84 (0.52, 1.35)	0.47
Quartile 4 (4.3-10.0)	60	74	0.67 (0.41, 1.11)	0.12
P-trend[3]				0.11
Model B[4]				
Quartile 1 (0.4-3.1)	85	75	Reference	--
Quartile 2 (3.1-3.6)	80	74	1.07 (0.68, 1.69)	0.76
Quartile 3 (3.7-4.3)	72	74	0.92 (0.56, 1.51)	0.75
Quartile 4 (4.3-10.0)	60	74	0.79 (0.46, 1.33)	0.37
P-trend[3]				0.30
Model C[5]				
Quartile 1 (0.4-3.1)	85	75	Reference	--
Quartile 2 (3.1-3.6)	80	74	1.07 (0.68, 1.69)	0.77
Quartile 3 (3.7-4.3)	72	74	0.92 (0.56, 1.52)	0.75
Quartile 4 (4.3-10.0)	60	74	0.78 (0.46, 1.33)	0.37
P-trend[3]				0.30

[1] *P*-value produced using the Wald test.

[2] Model A: Accounting for matching factors only.

[3] *P*-trend modeling the median of each quartile as a continuous variable, testing for linearity using the Wald test.

[4] Model B: Model A plus adjustment for parity/age at first birth (categorical: nulliparous, 1-2 pregnancies/age first birth<25, 1-2 pregnancies/age first birth ≥25, ≥3 pregnancies/age first birth <25, ≥3 pregnancies/age first birth≥25), family history of breast cancer (categorical: yes, no), history of biopsy-confirmed benign breast disease (categorical: yes, no), BMI at age 18 (continuous: kg/m^2), and average physical activity from 1989, 1991, and 1997 questionnaires (continuous: Metabolic Equivalent of Task [MET]-hrs/week).

[5] Model C: Model B plus adjustment for age at menarche (continuous: years), ever breastfed (categorical: yes, no), weight change between age 18 and blood collection (continuous: kg), and average alcohol consumption from 1991 and 1995 questionnaires (continuous: grams/day).

Abbreviations: CI=confidence interval; BMI=body mass index.

Table 3.

Associations between pre-diagnostic plasma osteoprotegerin quartiles and invasive breast cancer, stratified by reproductive, anthropometric, hormonal, and tumor characteristics: results from a matched case-control study nested within the Nurses' Health Study II.[1]

	Menopausal Status at Diagnosis/Index Date[2]					
	Premenopausal			Postmenopausal		
Osteoprotegerin (pmol/L)	No. of Cases	No. of Controls	Odds Ratio (95% CI)[3]	No. of Cases	No. of Controls	Odds Ratio (95% CI)[3]
Quartile 1 (0.4-3.1)	63	55	Reference	17	15	Reference
Quartile 2 (3.1-3.6)	60	55	1.05 (0.61, 1.79)	12	13	0.75 (0.22, 2.59)
Quartile 3 (3.7-4.3)	56	54	0.99 (0.58, 1.70)	11	12	0.99 (0.27, 3.63)
Quartile 4 (4.3-10.0)	39	50	0.72 (0.40, 1.31)	13	17	0.82 (0.24, 2.86)
P-trend[4]			0.29			0.86
P-interaction[5]						0.95
	BMI at Blood Collection					
	BMI < 25kg/m^2			BMI ≥ 25kg/m^2		
Quartile 1 (0.4-3.1)	46	45	Reference	39	30	Reference
Quartile 2 (3.1-3.6)	62	48	1.47 (0.81, 2.70)	18	26	0.45 (0.19, 1.07)
Quartile 3 (3.7-4.3)	44	44	1.05 (0.56, 1.98)	28	30	0.58 (0.26, 1.30)
Quartile 4 (4.3-10.0)	31	44	0.91 (0.46, 1.81)	29	30	0.63 (0.28, 1.42)
P-trend[4]			0.54			0.34
P-interaction[5]						0.21
	Luteal Estradiol at Blood Collection[6]					
	< Median (124.4 pg/mL)			≥ Median (124.4 pg/mL)		
Quartile 1 (0.4-3.1)	25	20	Reference	38	32	Reference
Quartile 2 (3.1-3.6)	30	26	1.04 (0.43, 2.55)	33	30	0.95 (0.46, 1.96)
Quartile 3 (3.7-4.3)	21	35	0.47 (0.19, 1.19)	32	21	1.06 (0.48, 2.34)
Quartile 4 (4.3-10.0)	16	29	0.36 (0.13, 1.00)	26	25	0.88 (0.40, 1.96)
P-trend[4]			0.02			0.82

Osteoprotegerin (pmol/L)	Menopausal Status at Diagnosis/Index Date[2]					
	Premenopausal			Postmenopausal		
	No. of Cases	No. of Controls	Odds Ratio (95% CI)[3]	No. of Cases	No. of Controls	Odds Ratio (95% CI)[3]
P-interaction[5]						0.17
	Luteal Progesterone at Blood Collection[6]					
	< Median (1403.3 ng/dL)			≥ Median (1403.3 ng/dL)		
Quartile 1 (0.4-3.1)	36	38	Reference	30	22	Reference
Quartile 2 (3.1-3.6)	32	27	1.33 (0.61, 2.91)	37	32	0.81 (0.36, 1.81)
Quartile 3 (3.7-4.3)	24	28	0.92 (0.41, 2.05)	33	33	0.78 (0.35, 1.74)
Quartile 4 (4.3-10.0)	18	25	0.71 (0.30, 1.69)	26	31	0.64 (0.28, 1.48)
P-trend[4]			0.39			0.31
P-interaction[5]						0.84
	Percent Mammographic Density					
	< Median (43.3%)			≥ Median (43.3%)		
Quartile 1 (0.4-3.1)	27	31	Reference	29	27	Reference
Quartile 2 (3.1-3.6)	19	29	1.23 (0.51, 2.94)	35	21	1.84 (0.81, 4.16)
Quartile 3 (3.7-4.3)	22	28	1.06 (0.46, 2.45)	29	27	1.02 (0.45, 2.34)
Quartile 4 (4.3-10.0)	18	18	1.18 (0.45, 3.09)	25	31	0.86 (0.37, 2.00)
P-trend[4]			0.80			0.42
P-interaction[5]						0.48
	Tumor Estrogen Receptor (ER) Status					
	ER +			ER −		
Quartile 1 (0.4-3.1)	66	75	Reference	19	75	Reference
Quartile 2 (3.1-3.6)	63	74	1.08 (0.66, 1.78)	12	74	0.67 (0.29, 1.56)
Quartile 3 (3.7-4.3)	57	74	0.99 (0.60, 1.64)	13	74	0.73 (0.32, 1.66)
Quartile 4 (4.3-10.0)	45	74	0.77 (0.45, 1.31)	12	74	0.77 (0.33, 1.80)
P-trend[4]			0.31			0.57
P-heterogeneity[7]						0.99

Osteoprotegerin (pmol/L)	Menopausal Status at Diagnosis/Index Date[2]					
	Premenopausal			Postmenopausal		
	No. of Cases	No. of Controls	Odds Ratio (95% CI)[3]	No. of Cases	No. of Controls	Odds Ratio (95% CI)[3]
	Tumor Progesterone Receptor (PR) Status					
	PR +			PR −		
Quartile 1 (0.4-3.1)	58	75	Reference	24	75	Reference
Quartile 2 (3.1-3.6)	54	74	1.07 (0.64, 1.79)	23	74	1.08 (0.54, 2.19)
Quartile 3 (3.7-4.3)	54	74	1.08 (0.65, 1.82)	16	74	0.73 (0.34, 1.54)
Quartile 4 (4.3-10.0)	41	74	0.80 (0.46, 1.39)	15	74	0.77 (0.35, 1.66)
P-trend[4]			0.46			0.36
P-heterogeneity[7]						0.61

[1] Cases and controls were matched on case diagnosis date, age at blood collection (+/− 2 years), time of blood collection (month [+/− 1 month] and time of day [+/− 2 hours]), fasting at blood collection (<2, 2-4, 5-7, 8-11, ≥12 hours since last meal), menopausal status at blood collection and in the questionnaire cycle before cancer diagnosis/control index date (premenopausal, postmenopausal, unknown), and self-reported race/ethnicity (white, non-white). In addition, women who gave a blood sample timed in the menstrual cycle (n=470) were matched on luteal day.

[2] Menopausal status in the questionnaire cycle immediately preceding breast cancer diagnosis or control index date.

[3] Odd ratios produced using unconditional logistic regression adjusted for matching factors and *a priori* potential confounders: age at menarche (continuous: years); parity/age at first birth (categorical: nulliparous, 1-2 pregnancies/age first birth<25, 1-2 pregnancies/age first birth ≥25, ≥3 pregnancies/age first birth <25, ≥3 pregnancies/age first birth≥25); ever breastfed (categorical: yes, no); family history of breast cancer (categorical: yes, no); history of biopsy-confirmed benign breast disease (categorical: yes, no); BMI at age 18 (continuous: kg/m^2); weight change between age 18 and blood collection (continuous: kg); average alcohol consumption from 1991 and 1995 questionnaires (continuous: grams/day); and average physical activity from 1989, 1991, and 1997 questionnaires (continuous: Metabolic Equivalent of Task [MET]-hrs/week).

[4] *P*-trend modeling the median of each quartile as a continuous variable, testing for linearity using the Wald test.

[5] *P*-interaction for multiplicative effect modification, assessed using a likelihood ratio test comparing models with vs. without an interaction term between the stratification variable and OPG (quartiles).

[6] Luteal estradiol and luteal progesterone measured from the same plasma sample as osteoprotegerin.

[7] *P*-heterogeneity assessed using unconditional nominal polytomous logistic regression adjusted for matching factors and confounders, testing for heterogeneity using the Wald test and allowing the effects of covariates to vary by tumor characteristics.

Abbreviations: CI=confidence interval; LRT=likelihood ratio test; BMI=body mass index; ER=estrogen receptor; PR=progesterone receptor.