Research Article

Cancer Epidemiology, Biomarkers & Prevention

Bioactive Prolactin Levels and Risk of Breast Cancer: A Nested Case–Control Study

Shelley S. Tworoger[1,2], Megan S. Rice[1,2], Bernard A. Rosner[1,3], Yvonne B. Feeney[4], Charles V. Clevenger[5], and Susan E. Hankinson[1,2,6]

Abstract

Background: Prolactin is a lactogenic hormone associated with breast cancer risk in prospective studies, which used immunoassays. The immunoassay captures multiple isoforms and may not fully reflect the biologic activity of prolactin relevant to breast carcinogenesis.

Methods: We considered plasma bioactive prolactin levels measured by the Nb2 lymphoma cell bioassay, which is sensitive to the somatolactogenic activity of prolactin and growth hormone, within a nested case–control study of invasive breast cancer in the Nurses' Health Studies (NHS/NHSII). We also considered associations with breast cancer risk factors.

Results: We had bioassay measures on 1,329 cases and 1,329 controls. Bioassay levels were inversely associated with parity (4+ vs. 0 children = −18%, $P = 0.01$), body mass index (30+ vs. <22 kg/m^2 = −16%, $P < 0.01$), and age at menopause (53+ vs. 48 years = −18%, $P = 0.03$) and positively with family history of breast cancer (yes vs. no = 14%, $P < 0.01$). The relative risk (RR) comparing the top versus bottom quartile of bioassay levels was 1.19 [95% confidence intervals (CI), 0.94–1.51; $P_{trend} = 0.18$]. The association was suggestively stronger for postmenopausal (RR = 1.36; 95% CI, 0.93–1.98; $P_{trend} = 0.12$) versus premenopausal women (RR = 0.99; 95% CI, 0.71–1.37; $P_{trend} = 0.71$). There was an association for cases diagnosed <4 years after blood draw (RR = 2.66; 95% CI, 1.45–4.89; $P_{trend} < 0.01$), but not for cases diagnosed later. We did not observe differential associations by estrogen receptor status or other tumor characteristics.

Conclusions: Our results show similar associations for prolactin levels measured by bioassay and by immunoassay with both breast cancer risk factors and risk.

Impact: Future work examining risk prediction model of breast cancer can use the immunoassay to accurately characterize risk.

Cancer Epidemiol Biomarkers Prev; 24(1); 73–80. ©2014 AACR.

Introduction

Prolactin is a lactogenic hormone that has been associated with breast cancer risk primarily in postmenopausal women (1–3). This hormone, which is involved in many biologic processes, including breast development and lactation (4), has a range of actions in part due to substantial posttranslational modifications (5). For example, glycosylated prolactin has a higher metabolic clearance rate and lower biologic activity due to reduced binding to receptors than the nonglycosylated form (6–8), and high-molecular weight isoforms (e.g., multimers, IgG complexes) tend to have lower biologic activity than the monomer (6, 9, 10). Furthermore, in mammary epithelial cells, unmodified prolactin may promote growth, while a phosphorylated isoform seems to inhibit growth (11–13). Prior epidemiologic studies primarily have used immunoassays to measure prolactin concentrations, these studies generally observed a 30% increase in risk comparing postmenopausal women with the highest versus lowest levels. However, immunoassays assess multiple isoforms and thus may not fully reflect the biologic activity of prolactin (7) that is relevant to breast carcinogenesis. Therefore, we considered bioactive prolactin levels as measured by the Nb2 lymphoma cell bioassay, which is a sensitive measure of the somatolactogenic activity of prolactin and growth hormone in plasma. Although the assay is highly specific to prolactin and growth hormone activity, between 78% and 95% of the activity measured by the bioassay is due to prolactin and measures of prolactin activity alone and total somatolactogenic activity are highly correlated (10, 14, 15). Furthermore, there is little epidemiologic evidence to support that growth hormone is associated with breast cancer risk (16).

Although data are limited, the bioassay may better reflect the portion of prolactin that is biologically important for breast cancer risk. In several small studies, circulating measures of bioactive prolactin, but not prolactin assessed by immunoassay, were 33% higher in breast cancer cases versus controls (10), were 2- to 3-fold higher in cases of benign breast disease versus controls (17–19), or among women with versus without a family history of breast cancer (17, 20). Because no study has prospectively evaluated risk of breast cancer with bioactive prolactin levels, we conducted a nested case–control study of breast cancer within the Nurses' Health Study (NHS) and NHSII measuring bioactive prolactin levels in plasma using the Nb2 lymphoma cell bioassay, additionally considering associations between the bioassay and breast cancer risk factors among controls.

[1]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. [2]Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts. [3]Department of Biostatistics, Harvard School of Public Health, Boston, Massachusetts. [4]Department of Pathology, Northwestern University, Chicago, Illinois. [5]Department of Pathology, Virginia Commonwealth University, Richmond, Virginia. [6]Division of Biostatistics and Epidemiology, School of Public Health and Health Sciences, University of Massachusetts, Amherst, Massachusetts.

Corresponding Author: Shelley S. Tworoger, Brigham and Women's Hospital and Harvard Medical School, 181 Longwood Avenue, 3rd Floor, Boston, MA 02115. Phone: 617-525-2087; Fax: 617-525-2008; E-mail: nhsst@channing.harvard.edu

doi: 10.1158/1055-9965.EPI-14-0896

Materials and Methods

The NHS was established in 1976 when 121,700 U.S. female registered nurses, ages 30 to 55 years, completed and returned a baseline questionnaire; similarly, NHSII began in 1989 with 116,430 female registered nurses (ages 25–42 years at baseline). In both cohorts, women consented to participate and have completed additional questionnaires every 2 years since study inception to update exposure and disease status.

In 1989–1990, 32,826 NHS participants provided a heparinized blood sample with a short questionnaire; women were 43 to 70 years old at blood collection. Details have been described previously (21), but briefly, women were mailed a collection kit, collected the sample, and shipped back the specimen with an ice pack to our laboratory via overnight courier. Samples were processed into plasma, red blood cells, and white blood cell components and have been stored in alarmed liquid nitrogen freezers (<−130°C) since collection. In addition, 186 postmenopausal women not using hormone therapy (HT) provided two more samples each one year apart (22). Similarly, 29,611 NHS participants (ages 32–54 years old) provided heparinized blood samples from 1996 to 1999 (23). Briefly, those women that were still cycling and had not been pregnant, taken hormones, or lactated within the prior 6 months provided two blood samples, one on the third to fifth day of the menstrual cycle (follicular) and one on the seventh to ninth day before the anticipated start date of the next cycle (luteal). Follicular plasma was aliquoted by the participant and frozen until the luteal sample was drawn. In addition, 236 women provided two more sets of follicular and luteal samples each one year apart (24). All other women (n = 11,090) provided a single, untimed sample. Luteal and untimed samples were processed as in the NHS collection. Follow-up of the participants in these cohorts is >95%. This study was approved by the Committee on the Use of Human Subjects in Research at the Brigham and Women's Hospital (Boston, MA).

We considered a woman to be premenopausal if (i) she gave timed samples, (ii) her periods had not ceased, or (iii) she had a hysterectomy with at least one ovary remaining and was 47 years or younger (nonsmokers) or 45 years or younger (smokers). We considered a woman to be postmenopausal if (i) her natural menstrual periods had ceased permanently, (ii) she had a bilateral oophorectomy, or (iii) she had a hysterectomy with at least one ovary remaining and was 56 years or older (nonsmokers) or 54 years or older (smokers). The remaining women, most of whom had a simple hysterectomy and were 48 to 55 years old, were considered to have an unknown menopausal status.

Cases were matched to one control on cohort (NHS and NHSII), menopausal status at baseline and diagnosis (premenopausal, postmenopausal, unknown), age (±2 years), month of blood collection (±1 month), time of day of blood draw (in 2-hour increments), fasting status (<8 vs. ≥8 hours), HT use at blood draw if postmenopausal (yes vs. no), and luteal day of blood draw (date of next period minus date of blood draw) if providing a timed sample. To assess within person stability over time, we conducted assays among 68 postmenopausal women and 105 premenopausal women with luteal samples (n = 33 had ample follicular sample for the assay) who gave three blood specimens. We also assessed the reproducibility of the assay with delayed processing, mimicking our blood collection methods, among 15 local donors, comparing blood processed immediately, versus 24 or 48 hours after collection (delayed processing samples kept with an ice pack).

Laboratory assays

For NHS, we assayed invasive cases diagnosed between June 1, 1992 and June 1, 2000—cases diagnosed within 2 years of blood collection were excluded to preserve their remaining sample volume for future early detection studies. For NHSII, we assayed invasive cases diagnosed after blood collection but before June 1, 2009. The Nb2 bioassay for biologically active somatolactogens was performed as previously described on these cases and controls (25, 26). Briefly, 24 hours before bioassay, $5 \times 10^4/cm^2$ cells were placed into a somatolactogen-deficient medium (DMEM-ITS). The bioassay was initiated by introducing dilutions of the plasma sample in quadruplicate; 48 hours later, proliferation was assessed by 3H-thymidine incorporation. Parallel cultures were stimulated with defined concentrations of recombinant prolactin (0–5 ng/mL, NIDDK) as controls. After growth factor restriction, Nb2 cells were cultured in the presence and absence of serum to establish baselines, in addition to varying concentrations of prolactin to establish the growth curves. The sensitivity of the assay was 3 pg/mL. Case–control samples were assayed in duplicate in 104 batches (NHS: 65 batches, NHSII: 39 batches) consisting of approximately 25 to 35 women each. Average within batch coefficients of variation (CV) were calculated either using blinded quality control (QC) samples (n = 77 batches) or using the average CV of the duplicate samples within a batch if blinded QCs were not available (n = 27 batches). CVs ranged from 0.3% to 17.3% for 97 of the batches (mean = 7.0%). CVs for seven batches were above 20% (range, 21.5–26.6); thus, we conducted a sensitivity analysis excluding these batches. The between batch CV was 40%. We also measured prolactin using a microparticle enzyme immunoassay on all the samples with the Nb2 bioassay except for NHSII cases diagnosed from 2003 to 2007 and matched controls who were postmenopausal at blood draw (for cost-saving reasons, n = 65; ref. 2). The limit of detection was 0.6 ng/mL and no samples were below the limit of detection; the average CV was 7.8% (SD = 3.5%).

Case–control sets were assayed together as were follicular and luteal samples (NHSII), ordered randomly within a set, and labeled with unique IDs. Case–control sets were randomly ordered within a batch, although NHS and NHSII samples were assayed in separate batches. Among NHSII women with timed samples, we measured both the follicular and luteal sample for the prolactin immunoassay as the average of these two measures provided the highest intraclass correlation (ICC) over 3 years (24). Only the luteal sample was measured for the bioassay as plasma volume of the follicular sample was limited and averaging the follicular and luteal value did not improve the ICC (see Results).

Statistical analysis

Because of the assay variation between laboratory batches over time, we recalibrated both immunoassay and bioassay levels from all batches to have a comparable distribution to an average batch (27). We assumed that all batches combined represented an average batch. We then regressed prolactin immunoassay or bioassay levels on indicator variables for batch as well as potential predictors of prolactin levels including age, case–control status, menopausal status, family history of breast cancer, age at

menopause, early life body size, body mass index (BMI) at blood draw, fasting status, time of blood collection, parity, history of HT use, and use of antidepressants and steroids at the time of blood collection. Levels were then recalibrated based on the coefficients for each batch and the average of the batch coefficients. We classified the recalibrated values into quartiles based on the overall distribution in controls.

ICCs in the pilot studies were calculated by dividing the between-person variance component by the sum of the between- and within-person variance components obtained from a mixed model, with participant ID as the random effect. ICCs were assessed on ln-transformed assay values.

We used linear regression adjusting for matching factors among controls only to evaluate the association of prolactin bioassay levels with reproductive and hormonal factors previously associated with breast cancer risk. We evaluated the following factors assessed at or near blood collection in a multivariate model: age (<45, 45–59, 50–54, 55–59, 60+ years), BMI (<22, 22–24.9, 25–29.9, 30+ kg/m^2), parity (0, 1, 2, 3, 4+ children), age at first birth among parous women (<25, 25–29, 30+ years), breastfeeding among parous women (never, 1–11, 12+ months), family history of breast cancer (yes, no), personal history of benign breast disease (yes, no), antidepressant use (yes, no), alcohol intake (0, <5, 5+ g/day), age at menarche (<12, 12, 13, 14+ years), average childhood body size at ages 5 and 10 years based on a 9-level pictogram (ref. 28; 1, >1–2, >2–3, >3), menopausal status/HT use (premenopausal, postmenopausal/current HT use, postmenopausal/never or past HT use), and age at menopause among postmenopausal women (<48, 48–50, 51–52, 53+ years). These variables were assessed on the questionnaire completed at the time of blood collection or on the biennial questionnaires.

Relative risks (RR) and 95% confidence intervals (CI) of breast cancer were determined using conditional logistic regression comparing quartiles of prolactin bioassay concentrations (29). Tests for trend were modeled using quartile medians and assessed using the Wald statistic. Models were adjusted for BMI (continuous), childhood body size (continuous), age at menarche (<12, 12, 13, 14+ years), history of benign breast disease (yes, no), family history of breast cancer (yes, no), antidepressant use (yes, no), age at menopause (continuous, with an indicator for premenopausal), and alcohol intake (0, <5, 5+ g/day). We considered separate adjustment for parity/age at first birth (nulliparous, 1–2 children/<25, 1–2 children/25–29, 1–2 children/30+, 3+ children/<25, 3+ children 25+) and breastfeeding (none, <12 months, 12+ months) as this may be part of the biologic pathway through which prolactin affects breast cancer (30).

On the basis of our prior results using the immunoassay (2), we investigated whether the bioassay/breast cancer association varied by menopausal status at blood collection by including a multiplicative interaction term between menopausal status and the prolactin quartile median variable, using the Wald test as well as assessing associations by time between blood draw and diagnoses/reference date (<4, 4–7.9, 8+ years). We additionally assessed associations by tumor characteristics differentially associated with the immunoassay using polytomous logistic regression including estrogen (ER) and progesterone (PR) receptor status, ductal versus lobular, HER2$^+$ tumors, tumor size, and lymph node metastasis status, adjusting for covariates as described above and matching factors [age at blood draw (continuous), date of blood draw (continuous), time since last meal (≥8 vs. <8 hours), time of day of blood draw (midnight–6 am, 6–8 am, 8 am–midnight), menopausal/HT status (premenopausal, postmenopausal/current HT use, postmenopausal/never or past HT use), and cohort (NHS, NHSII)]. We also considered whether three other metrics combining information about the bioassay and immunoassay were associated with breast cancer risk, including the ratio of the bioassay to immunoassay, a score that summed the decile ranking for each of the assays, or the cross-classification of the two assays, as prior studies of the bioassay suggested that this may reflect the nonmonomeric prolactin forms (15, 17, 19) and in one study, the bioassay:immunoassay ratio was higher in breast cancer cases versus controls (10).

All P values were two sided and were considered statistically significant if <0.05. All analyses were conducted using SAS, version 9.3 (SAS Institute Inc.).

Results

We conducted pilot studies to assess the stability of prolactin bioactivity with delayed processing of the sample for up to 48 hours as well as the within person stability of bioactive prolactin over 3 years in premenopausal and postmenopausal women separately. The ICC comparing samples processed immediately versus 24 or 48 hours after collection was 0.87, suggesting that bioactivity of prolactin is not substantially altered by delayed processing. When examining within person stability over 3 years, we observed an ICC of 0.63 for postmenopausal women, and in premenopausal women, an ICC of 0.66 for the follicular phase, 0.63 for the luteal phase, and 0.64 for the average of the follicular and luteal phases. The correlation between the bioassay and immunoassay was 0.57.

We had assay results available on 1,329 cases and 1,329 controls, about 59% of which came from the NHS (Table 1). Cases were more likely to be nulliparous, have a family history of breast cancer and a personal history of benign breast disease than controls.

Among controls, parity was inversely associated with bioactive prolactin levels ($P = 0.01$; Table 2); nulliparous women had the highest levels (least square mean = 10.8), those with one child had intermediate levels (mean = 9.5), those with two or more children had the lowest levels (mean ranged from 8.8–8.9). Those with 4+ children had 18% lower bioactive prolactin than nulliparous women. In addition, women with a family history of breast cancer had 14% higher bioactive prolactin levels than those without a family history ($P < 0.01$). Both age at menopause ($P = 0.03$; percent difference comparing 53+ with <48 years = −18%) and BMI ($P < 0.01$, percent difference comparing 30+ with <22 kg/m^2 = −16%) were inversely associated with bioactive prolactin levels. Premenopausal women had suggestively higher bioactive prolactin levels (least squared mean = 9.6, $P = 0.43$) than postmenopausal women using HT at blood draw (mean = 8.7) or postmenopausal women not using HT (mean = 8.4). Other factors, including age, age at first birth, breast feeding, history of benign breast disease, antidepressant use, alcohol use, age at menarche, and early life body size were not significantly associated with bioactive prolactin levels.

In age-adjusted analyses, bioactive prolactin was suggestively positively associated with breast cancer risk overall (RR, >11.2 vs. <5.7 ng/mL = 1.25; 95% CI, 0.97–1.53; $P_{trend} = 0.12$); however, the association was slightly attenuated in multivariate adjustment (comparable RR, 1.19; 95% CI, 0.94–1.51; $P_{trend} = 0.18$; Table 3). The association was possibly stronger ($P_{heterogeneity} = 0.55$) for

Table 1. Characteristics at blood collection of breast cancer cases and matched controls from the NHS (follow-up from 1994–2000) and NHSII (follow-up from 1999–2009)

	Cases ($N = 1,329$)	Controls ($N = 1,329$)
Mean (SD)		
Age at blood collection (y)[a]	52.3 (8.4)	52.3 (8.3)
BMI at blood collection (kg/m^2)	25.7 (4.0)	25.3 (3.8)
Childhood somatotype (average of ages 5 and 10)[b]	2.3 (1.2)	2.4 (1.3)
Age at menarche	12.4 (1.4)	12.6 (1.4)
Parity (among parous)	2.5 (1.6)	2.6 (1.6)
Age at first birth (among parous)	25.7 (4.0)	25.3 (3.8)
Age at menopause (among postmenopausal)	49.1 (4.9)	48.5 (5.4)
Nb2 prolactin bioassay (ng/mL)	9.3 (5.2)	9.1 (5.2)
Frequency (percentage)		
Nulliparous	180 (13.5)	135 (10.2)
Breastfeeding		
Never	338 (29.7)	401 (33.8)
1–11 mo	412 (36.2)	398 (33.6)
12+ mo	387 (34.0)	386 (32.6)
Benign breast disease	465 (35.0)	361 (27.2)
Family history of breast cancer	213 (16.0)	134 (10.1)
Alcohol use		
None	500 (38.5)	507 (38.9)
<5 g/d	436 (33.6)	476 (36.5)
5+ g/d	362 (27.9)	320 (24.6)
Antidepressant use	116 (8.7)	128 (9.6)
Menopausal status/HT use[a]		
Premenopausal	761 (53.9)	711 (53.5)
Postmenopausal, no HT	244 (18.4)	255 (19.2)
Postmenopausal, HT	369 (27.8)	363 (27.3)
Cohort[a]		
NHS	786 (59.1)	786 (59.1)
NHSII	543 (40.9)	543 (40.9)

[a]Matching factor.
[b]On the basis of a 9-level pictogram of various body shapes.

postmenopausal (comparable multivariate RR, 1.36; 95% CI, 0.93–1.98) than for premenopausal (comparable multivariate RR = 0.99; 95% CI, 0.71–1.37) women. Among postmenopausal women, current HT users had a suggestively stronger association in the top quartile versus bottom than those not using HT at blood draw, but the risk estimates for quartiles 2 and 3 were similar by HT use. Within the dataset on which we had bioassay results, the corresponding RRs for the prolactin immunoassay were 1.01 (95% CI, 0.79–1.30) for all women, 1.18 (95% CI, 0.80–1.72) for postmenopausal women, and 0.76 (95% CI, 0.51–1.12) for premenopausal women; these results are generally consistent with that observed in a much larger dataset from our prior analysis of the immunoassay (2), although not statistically significant due to the smaller sample size.

We also observed a difference ($P_{\text{heterogeneity}} = 0.02$) in the strength of the association by time between blood draw and diagnosis. A significant positive association was observed for cases diagnosed within 4 years of blood draw (comparable RR; 2.66, 95% CI, 1.45–4.89; $P_{\text{trend}} < 0.01$), but bioactive prolactin was not associated with cases diagnosed 4 to 8 ($P_{\text{trend}} = 0.78$) or 8+ years ($P_{\text{trend}} = 0.74$) after blood draw. The association differed neither by estrogen receptor (ER) status ($P_{\text{heterogeneity}} = 0.69$) nor by other tumor characteristics including progesterone receptor status, HER2 status, invasive versus *in situ*, ductal versus lobular, tumor size, or lymph node status (data not shown). Additional adjustment for parity/age at first birth, and breastfeeding slightly attenuated the results (data not shown). Furthermore, the ratio of bioassay:immunoassay prolactin levels, a score summing the deciles of each assay, or cross-classified bioassay/immunoassay levels were not associated with breast cancer risk (data not shown). Results were similar when excluding the seven batches with CVs > 20% (data not shown).

Discussion

This is the first prospective study examining the relationship of bioactive prolactin levels as assessed by the Nb2 bioassay and risk of breast cancer in over 1,300 cases and controls. Overall, we did not observe a clear relationship between prolactin measured using this assay and breast cancer risk, although there was a suggestive association for postmenopausal women and a significant positive relationship for women diagnosed within 4 years of blood draw. Furthermore, bioactive prolactin was associated in cross-sectional analyses with several known breast cancer risk factors, including parity, family history of breast cancer, age at menopause, and BMI.

Similar to what has been previously observed by ourselves and others in analyses of the prolactin immunoassay and breast cancer risk (1–3), there was about a 30% and 40% increase in risk of invasive postmenopausal breast cancer for women in the top versus bottom quartile of bioactive prolactin, but no association for premenopausal women. The lack of statistical significance for the postmenopausal group in this analysis likely reflects the smaller sample size than in our immunoassay study.

Our hypothesis was that the association would be substantially stronger for bioactive prolactin than for levels assessed by immunoassay because of the heterogeneity of circulating prolactin (6) and because the bioassay could more directly reflect the hormone's effect on cell proliferation. The observed similar or only

Table 2. Relationship between reproductive and hormonal factors assessed at or near blood collection with prolactin bioassay levels among 1,339 control women from the NHS and NHSII[a]

Variable	Sample size	Least square mean (95% CI) prolactin bioassay level	*P*, *F* test
Age, y			0.31
<45	273	9.0 (8.2–9.8)	
45–49	321	9.0 (8.3–9.7)	
50–54	247	8.6 (7.9–9.3)	
55–59	189	9.8 (9.0–10.7)	
60+	299	9.4 (8.5–10.3)	
BMI (kg/m^2)			<0.01
<22	323	9.6 (9.1–10.2)	
22–24.9	387	9.6 (9.2–10.1)	
25–29.9	393	8.8 (8.3–9.3)	
30+	225	8.1 (7.4–8.8)	
Parity			0.01
Nulliparous	135	10.8 (9.8–11.8)	
1	118	9.5 (8.5–10.4)	
2	406	8.9 (8.4–9.4)	
3	360	8.8 (8.3–9.4)	
4+	308	8.9 (8.3–9.5)	
Age at first birth, y[b]			0.45
<25	581	8.8 (8.4–9.2)	
25–29	438	9.1 (8.7–9.6)	
30+	160	8.8 (8.0–9.6)	
Breastfeeding (mo)[b]			0.71
0	401	8.9 (8.4– 9.4)	
1–11	398	9.1 (8.6–9.5)	
12+	386	8.8 (8.3–9.3)	
Family history of breast cancer			<0.01
No	1195	9.0 (8.7–9.3)	
Yes	134	10.3 (9.5–11.1)	
History of benign breast disease			0.45
No	968	9.1 (8.8–9.4)	
Yes	361	9.3 (8.8–9.8)	
Antidepressant use			0.67
No	1201	9.1 (8.9–9.4)	
Yes	128	9.0 (8.1–9.8)	
Alcohol (g/d)			0.78
None	507	9.1 (8.7– 9.6)	
<5	476	9.0 (8.6– 9.5)	
5+	320	9.3 (8.7– 9.8)	
Age at menarche, y			0.77
<12	270	9.2 (8.6– 9.8)	
12	386	8.9 (8.4– 9.4)	
13	397	9.3 (8.8– 9.7)	
14+	272	9.2 (8.6– 9.8)	
Average childhood body size at ages 5 and 10[c]			0.38
1	317	9.0 (8.5– 9.6)	
2	372	9.5 (9.0– 10.0)	
3	282	8.9 (8.3– 9.4)	
4+	311	9.0 (8.5– 9.6)	
Menopausal status/HT use			0.43
Premenopausal	711	9.6 (8.7– 10.5)	
Postmenopausal/HT user	363	8.7 (7.7– 9.8)	
Postmenopausal/no HT use	255	8.4 (7.3– 9.5)	
Age at natural menopause, y[d]			0.03
<48	168	8.5 (7.8– 9.2)	
48–50	172	8.1 (7.5– 8.8)	
51–52	146	8.5 (7.8– 9.2)	
53+	100	7.0 (6.2– 7.8)	

[a]Assessed using a multivariate mixed model including all of the above exposures as well as cohort (NHS, NHSII), time since last meal (≥8, <8 h), time of day of blood draw (midnight—6 am, 6 am—8 am, 8 am—midnight), and date of blood draw (continuous).
[b]Among parous women.
[c]On the basis of a 9-level pictogram of various body shapes.
[d]Among postmenopausal women only.

slightly stronger association for prolactin measured via the bioassay versus the immunoassay may be due to several reasons. First, the somatolactogenic receptors on the Nb2 rat lymphoma cell line may not reflect the receptors expressed in human breast tissue. Prolactin receptor has multiple (4) isoforms and the particular forms of prolactin that activate the receptors in breast tissue may differ from those in the Nb2 cell line. One study examined the correlation between the Nb2 assay and a similar assay using

Table 3. Relative risk[a] (95% CIs) of bioactive prolactin levels with invasive breast cancer risk among all women, by menopausal status, and time between blood draw and diagnosis/reference date in the NHS and NHSII

	Bioactive prolactin levels (ng/mL)				
	<5.7	5.7–<8.2	8.2–<11.2	≥11.2	P_{trend}
All women	301/332[b]	339/333	329/332	360/332	
	1.00 (Ref.)	1.12 (0.90–1.41)	1.10 (0.87–1.40)	1.19 (0.94–1.51)	0.18
Menopausal status/HT use					
Premenopausal	139/132	153/171	183/194	241/214	
	1.00 (Ref.)	0.81 (0.57–1.14)	0.83 (0.59–1.18)	0.99 (0.71–1.37)	0.71
Postmenopausal	162/200	186/162	146/138	119/118	
	1.00 (Ref.)	1.49 (1.07–2.06)	1.48 (1.03–2.12)	1.36 (0.93–1.98)	0.12
No HT use	83/103	83/65	46/45	32/42	
	1.00 (Ref.)	1.47 (0.90–2.41)	1.36 (0.75–2.47)	1.07 (0.55–2.09)	0.69
Current HT use	79/97	103/97	100/93	87/76	
	1.00 (Ref.)	1.43 (0.89–2.29)	1.45 (0.89–2.38)	1.45 (0.88–2.41)	0.19
Time between blood draw and diagnosis					
<4 y	57/68	71/84	71/82	107/72	
	1.00 (Ref.)	1.01 (0.58–1.74)	1.10 (0.62–1.93)	2.66 (1.45–4.89)	<0.01
4–8 y	129/149	164/140	134/131	139/146	
	1.00 (Ref.)	1.41 (0.99–2.00)	1.20 (0.83–1.73)	1.04 (0.73–1.49)	0.78
8+ y	115/115	104/109	124/119	114/114	
	1.00 (Ref.)	0.98 (0.66–1.46)	1.09 (0.72–1.65)	1.04 (0.70–1.50)	0.74
Estrogen receptor (ER) status					
ER^+	221/332	248/333	240/332	264/332	
	1.00 (Ref.)	1.12 (0.88–1.43)	1.06 (0.83–1.36)	1.15 (0.90–1.48)	0.35
ER^-	57/332	64/333	63/332	63/332	
	1.00 (Ref.)	1.12 (0.76–1.66)	1.08 (0.73–1.61)	1.07 (0.72–1.59)	0.84

[a]Conditional logistic regression adjusted for BMI (continuous), average childhood body size (continuous), age at menarche (<12, 12, 13, ≥14), personal history of benign breast disease (no, yes), family history of breast cancer (no, yes), antidepressant use (no, yes), age at menopause (continuous), and alcohol use (none, < 5 g/d, ≥ 5 g/d) for all analyses except by ER status, which was assessed by unconditional polytomous logistic regression adjusted for matching factors [age at blood draw (continuous), date of blood draw (continuous), time since last meal (≥8 vs. <8 h), time of day of blood draw (midnight–6 am, 6–8 am, 8 am–midnight), menopausal/HT status (premenopausal, postmenopausal/current HT use, postmenopausal/never or past HT use), and cohort (NHS, NHSII)], and the above covariates.
[b]*n*, cases/controls.

human prolactin receptor (31). When the predominant form of prolactin was monomeric, the two assays performed similarly; however, when high levels of macroprolactin were present, the Nb2 assay had higher levels than the human-based assay. Second, although the bioactivity assay is primarily influenced by prolactin, proliferation also can be stimulated by growth hormone (15, 26), although the concentrations of growth hormone must be very high to stimulate Nb2 cells and over 80% of the stimulation is due to prolactin (10, 14, 15). Notably, this assay is specific to prolactin and growth hormone as there is no cell proliferation observed when including antiprolactin and antigrowth hormone antibodies to the assay (15). We observed a correlation of 0.57 between the immunoassay and bioassay, which is slightly lower than that observed in prior studies ($r = \sim$0.73–0.95; refs. 15, 17, 19). This may reflect that we used a microparticle immunoassay as opposed to a radioimmunoassay in the other studies. Third, while the within assay CV was very good for the bioassay, in part because we ran samples in quadruplicate, the between assay CV was higher (∼40%). This may be due to the use of live cells, which cannot be standardized in the same way as chemical reagents. We corrected for this statistically using a batch recalibration technique designed specifically to address this type of heterogeneity (27), but some amount of measurement error may have attenuated the results.

Interestingly, as with the immunoassay (2), we observed a significantly stronger association between the bioassay and risk of disease occurring shortly after blood collection, with over a 2-fold risk of breast cancer comparing women with the highest versus lowest bioactive prolactin levels. This may reflect production of biologically active prolactin by the tumor itself, increasing circulating levels (32–34). Prolactin also may be involved in processes important for late-stage carcinogenesis, such as proliferation, cell migration, and angiogenesis (4, 35–40), and high levels, particularly of bioactive prolactin, could enhance those processes *in vivo*. Furthermore, prolactin has some within person variability over time suggesting that the association could attenuate with increasing time between blood draw and diagnosis. The immunoassay has an ICC of about 0.4 to 0.55 over 3 years (24, 41), but the bioassay has a higher ICC in the same datasets of around 0.63 to 0.66, which is similar to that for blood pressure and cholesterol (42). Unlike the immunoassay results, we did not observe stronger associations for ER^+ tumors either overall or in postmenopausal women (data not shown). The relatively small number of postmenopausal women with ER^- ($n = 98$) disease may have limited our ability to observe a differential relationship by ER status.

Similar to prior studies of the immunoassay (30, 43–46), we observed that parity, but not age at first birth or breast feeding, was associated with bioactive prolactin levels. Nulliparous women had significantly higher bioactive prolactin than parous women. Several lines of evidence suggest that pituitary prolactin secretion diminishes after pregnancy (47, 48). We also observed that women with a family history of breast cancer had higher bioactive prolactin levels; this is consistent with both previous bioassay (17, 20) and immunoassay (30, 43) results. It is possible that some heritable component of breast cancer risk also influences hormone levels (49). Prior small studies ($N < 52$) of bioactive prolactin in both blood and nipple aspirate reported higher levels in benign breast disease patients than controls; (17–19, 50) however, our much larger study did not observe such an association. Interestingly, we noted an inverse association between current BMI and bioactive prolactin, which is inconsistent with previous studies of the immunoassay (45, 51, 52) that have either

observed positive or no association with BMI. The reason for this association is unclear, but it is possible that this relationship may be driven in part by the influence of growth hormone on the bioassay. In one previous analysis in the NHSII, we observed that growth hormone was strongly inversely associated with BMI (53). Another possibility is that recent evidence suggests that prolactin can be produced and released by adipose tissue; however, cell culture studies of adipocytes from obese versus nonobese individuals suggest that prolactin release is substantially lower in obese women (54).

Overall, the results of this study are consistent with the hypothesis that prolactin is a risk factor for breast cancer. However, given that the association was largely similar for bioactive prolactin and prolactin measured by immunoassay and the lower inter-assay CVs for the immunoassay, future epidemiologic studies should consider using the immunoassay, which is less expensive and more high throughput. Additional research understanding the etiology of prolactin in breast carcinogenesis, particularly on late-stage effects, is warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.S. Tworoger, S.E. Hankinson
Development of methodology: S.S. Tworoger, C.V. Clevenger, S.E. Hankinson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.S. Tworoger, Y.B. Feeney, C.V. Clevenger, S.E. Hankinson
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.S. Tworoger, M.S. Rice, B.A. Rosner, S.E. Hankinson
Writing, review, and/or revision of the manuscript: S.S. Tworoger, M.S. Rice, B.A. Rosner, C.V. Clevenger, S.E. Hankinson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.V. Clevenger
Study supervision: S.S. Tworoger, S.E. Hankinson

Acknowledgments

The authors thank the following state cancer registries for their help: AL, AZ, AR, CA, CO, CT, DE, FL, GA, ID, IL, IN, IA, KY, LA, ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, and WY.

Grant Support

This work was supported by the NIH [R01 CA49449 (PI: S.E. Hankinson), R01 CA138580 (PI: S.S. Tworoger), P01 CA87969 (PI: Stampfer), R01 CA67262 (PI: S.E. Hankinson), R01 CA50385 (PI: Willett), and T32 CA09001 (PI: Stampfer)]. Also, this study was approved by the Connecticut Department of Public Health (DPH) Human Investigations Committee. Certain data used in this publication were obtained from the DPH.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 31, 2014; revised October 7, 2014; accepted October 7, 2014; published OnlineFirst October 14, 2014.

References

1. Manjer J, Johansson R, Berglund G, Janzon L, Kaaks R, Agren A, et al. Postmenopausal breast cancer risk in relation to sex steroid hormones, prolactin and SHBG (Sweden). Cancer Causes Control 2003;14:599–607.
2. Tworoger SS, Eliassen AH, Zhang X, Qian J, Sluss PM, Rosner BA, et al. A 20-year prospective study of plasma prolactin as a risk marker of breast cancer development. Cancer Res 2013;73:4810–9.
3. 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.
4. Clevenger CV, Furth PA, Hankinson SE, Schuler LA. The role of prolactin in mammary carcinoma. Endocr Rev 2003;24:1–27.
5. Sinha YN. Structural variants of prolactin: occurrence and physiological significance. Endocr Rev 1995;16:354–69.
6. Garcia M, Colombani-Vidal ME, Zylbersztein CC, Testi A, Marcos J, Arturi A, et al. Analysis of molecular heterogeneity of prolactin in human systemic lupus erythematosus. Lupus 2004;13:575–83.
7. Haro LS, Lee DW, Singh RN, Bee G, Markoff E, Lewis UJ. Glycosylated human prolactin: alterations in glycosylation pattern modify affinity for lactogen receptor and values in prolactin radioimmunoassay. J Clin Endocrinol Metab 1990;71:379–83.
8. Hoffmann T, Penel C, Ronin C. Glycosylation of human prolactin regulates hormone bioactivity and metabolic clearance. J Endocrinol Invest 1993;16:807–16.
9. Bulatov AA, Osipova TA, Makarovskaia EE. Comparison of biological activity of high molecular weight and low molecular weight forms of immunoreactive prolactin from human serum in cultured lymphoma NB2 cells. Vopr Med Khim 1996;42:234–9.
10. Maddox PR, Jones DL, Mansel RE. Prolactin and total lactogenic hormone measured by microbioassay and immunoassay in breast cancer. Br J Cancer 1992;65:456–60.
11. Kuo CB, Wu W, Xu X, Yang L, Chen C, Coss D, et al. Pseudophosphorylated prolactin (S179D PRL) inhibits growth and promotes beta-casein gene expression in the rat mammary gland. Cell Tissue Res 2002;309:429–37.
12. Schroeder MD, Brockman JL, Walker AM, Schuler LA. Inhibition of prolactin (PRL)-induced proliferative signals in breast cancer cells by a molecular mimic of phosphorylated PRL, S179D-PRL. Endocrinology 2003;144:5300–7.
13. Wu W, Chen YH, Ueda E, Tan D, Bartolini P, Walker AM. Different forms of prolactin have opposing effects on the expression of cell cycle regulatory proteins in differentiated mammary epithelial cells. Oncol Res 2006;16:75–84.
14. Lawson DM, Sensui N, Haisenleder DH, Gala RR. Rat lymphoma cell bioassay for prolactin: observations on its use and comparison with radioimmunoassay. Life Sci 1982;31:3063–70.
15. Tanaka T, Shiu RP, Gout PW, Beer CT, Noble RL, Friesen HG. A new sensitive and specific bioassay for lactogenic hormones: measurement of prolactin and growth hormone in human serum. J Clin Endocrinol Metab 1980;51:1058–63.
16. Schernhammer ES, Holly JM, Hunter DJ, Pollak MN, Hankinson SE. Insulin-like growth factor-I, its binding proteins (IGFBP-1 and IGFBP-3), and growth hormone and breast cancer risk in The Nurses Health Study II. Endocr Relat Cancer 2006;13:583–92.
17. Rose DP, Berke B, Cohen LA. Serum prolactin and growth hormone determined by radioimmunoassay and a two-site immunoradiometric assay: comparison with the Nb2 cell bioassay. Horm Metab Res 1988;20:49–53.
18. Rose DP, Berke B, Cohen LA, Lahti H. A comparison of serum and breast duct fluid-immunoassayable prolactin and growth hormone with bioassayable lactogenic hormones in healthy women and patients with cystic breast disease. Cancer 1987;60:2761–5.
19. Rose DP, Cohen LA, Berke B, Boyar AP. Effect of a low-fat diet on hormone levels in women with cystic breast disease. II. Serum radioimmunoassayable prolactin and growth hormone and bioactive lactogenic hormones. J Natl Cancer Inst 1987;78:627–31.
20. Love RR, Rose DP. Elevated bioactive prolactin in women at risk for familial breast cancer. Eur J Cancer Clin Oncol 1985;21:1553–4.
21. Hankinson SE, Willett WC, Manson JE, Hunter DJ, Colditz GA, Stampfer MJ, et al. Alcohol, height, and adiposity in relation to estrogen and prolactin levels in postmenopausal women. J Natl Cancer Inst 1995;87:1297–302.
22. Hankinson SE, Manson JE, Spiegelman D, Willett WC, Longcope C, Speizer FE. Reproducibility of plasma hormone levels in postmenopausal women over a 2–3-year period. Cancer Epidemiol Biomarkers Prev 1995;4:649–54.

23. Tworoger SS, Sluss P, Hankinson SE. Association between plasma prolactin concentrations and risk of breast cancer among predominately premenopausal women. Cancer Res 2006;66:2476–82.
24. Missmer SA, Spiegelman D, Bertone-Johnson ER, Barbieri RL, Pollak MN, Hankinson SE. Reproducibility of plasma steroid hormones, prolactin, and insulin-like growth factor levels among premenopausal women over a 2- to 3-year period. Cancer Epidemiol Biomarkers Prev 2006;15:972–8.
25. Clevenger CV, Chang WP, Ngo W, Pasha TL, Montone KT, Tomaszewski JE. Expression of prolactin and prolactin receptor in human breast carcinoma. Evidence for an autocrine/paracrine loop. Am J Pathol 1995;146:695–705.
26. Gout PW, Beer CT, Noble RL. Prolactin-stimulated growth of cell cultures established from malignant Nb rat lymphomas. Cancer Res 1980;40: 2433–6.
27. Rosner B, Cook N, Portman R, Daniels S, Falkner B. Determination of blood pressure percentiles in normal-weight children: some methodological issues. Am J Epidemiol 2008;167:653–66.
28. Stunkard AJ, Sorensen T, Schulsinger F. Use of the Danish Adoption Register for the study of obesity and thinness. In: The genetics of neurological and psychiatric disorders. edn. Edited byKety SS, Rowland LP, Sidman SW, Mathysee SW. New York City: Raven Press; 1983: 115–20.
29. Rosner BA. Fundamentals of Biostatistics, 6 edn. Belmont, CA. Duxbury Press; 2005.
30. Eliassen AH, Tworoger SS, Hankinson SE. Reproductive factors and family history of breast cancer in relation to plasma prolactin levels in premenopausal and postmenopausal women. Int J Cancer 2007;120:1536–41.
31. Leanos-Miranda A, Cardenas-Mondragon G, Rivera-Leanos R, Ulloa-Aguirre A, Goffin V. Application of new homologous *in vitro* bioassays for human lactogens to assess the actual bioactivity of human prolactin isoforms in hyperprolactinaemic patients. Clin Endocrinol (Oxf) 2006;65:146–53.
32. Bhatavdekar JM, Patel DD, Shah NG, Vora HH, Suthar TP, Ghosh N, et al. Prolactin as a local growth promoter in patients with breast cancer: GCRI experience. Eur J Surg Oncol 2000;26:540–7.
33. Ginsburg E, Vonderhaar BK. Prolactin synthesis and secretion by human breast cancer cells. Cancer Res 1995;55:2591–5.
34. Lachelin GC, Yen SC, Alksne JF. Hormonal changes following hypophysectomy in humans. Obstet Gynecol 1977;50:333–9.
35. Aksamitiene E, Achanta S, Kolch W, Kholodenko BN, Hoek JB, Kiyatkin A. Prolactin-stimulated activation of ERK1/2 mitogen-activated protein kinases is controlled by PI3-kinase/Rac/PAK signaling pathway in breast cancer cells. Cell Signal 2011;23:1794–805.
36. Jacobson EM, Hugo ER, Borcherding DC, Ben-Jonathan N. Prolactin in breast and prostate cancer: molecular and genetic perspectives. Discov Med 2011;11:315–24.
37. Liby K, Neltner B, Mohamet L, Menchen L, Ben-Jonathan N. Prolactin overexpression by MDA-MB-435 human breast cancer cells accelerates tumor growth. Breast Cancer Res Treat 2003;79:241–52.
38. Maus MV, Reilly SC, Clevenger CV. Prolactin as a chemoattractant for human breast carcinoma. Endocrinology 1999;140:5447–50.
39. Reuwer AQ, Nowak-Sliwinska P, Mans LA, van der Loos CM, von der Thusen JH, Twickler MT, et al. Functional consequences of prolactin signaling in endothelial cells: a potential link with angiogenesis in pathophysiology? J Cell Mol Med 2012;16:2035–48.
40. Struman I, Bentzien F, Lee H, Mainfroid V, D'Angelo G, Goffin V, et al. Opposing actions of intact and N-terminal fragments of the human prolactin/growth hormone family members on angiogenesis: an efficient mechanism for the regulation of angiogenesis. Proc Natl Acad Sci U S A 1999;96:1246–51.
41. Tworoger SS, Eliassen AH, Rosner B, Sluss P, Hankinson SE. Plasma prolactin concentrations and risk of postmenopausal breast cancer. Cancer Res 2004;64:6814–9.
42. Willett WC. Nutritional Epidemiology, vol., 2nd edn. New York: Oxford University Press; 1998.
43. Clendenen TV, Arslan AA, Lokshin AE, Liu M, Lundin E, Koenig KL, et al. Circulating prolactin levels and risk of epithelial ovarian cancer. Cancer Causes Control 2013;24:741–8.
44. Wang DY, de Stavola BL, Bulbrook RD, Allen DS, Kwa HG, Verstraeten AA, et al. The relationship between blood prolactin levels and risk of breast cancer in premenopausal women. Eur J Cancer Clin Oncol 1987;23: 1541–8.
45. Wang DY, de Stavola BL, Bulbrook RD, Allen DS, Kwa HG, Verstraeten AA, et al. The permanent effect of reproductive events on blood prolactin levels and its relation to breast cancer risk: a population study of postmenopausal women. Eur J Cancer Clin Oncol 1988;24:1225–31.
46. Yu MC, Gerkins VR, Henderson BE, Brown JB, Pike MC. Elevated levels of prolactin in nulliparous women. Br J Cancer 1981;43:826–31.
47. Espinosa de los Monteros A, Cornejo J, Parra A. Differential prolactin response to oral metoclopramide in nulliparous versus parous women throughout the menstrual cycle. Fertil Steril 1991;55:885–9.
48. Musey VC, Collins DC, Musey PI, Martino-Saltzman D, Preedy JR. Long-term effect of a first pregnancy on the secretion of prolactin. N Engl J Med 1987;316:229–34.
49. Lee SA, Haiman CA, Burtt NP, Pooler LC, Cheng I, Kolonel LN, et al. A comprehensive analysis of common genetic variation in prolactin (PRL) and PRL receptor (PRLR) genes in relation to plasma prolactin levels and breast cancer risk: the multiethnic cohort. BMC Med Genet 2007; 8:72.
50. Rose DP. Hormones and growth factors in nipple aspirates from normal women and benign breast disease patients. Cancer Detect Prev 1992;16: 43–51.
51. Kwa HG, Cleton F, Bulbrook RD, Wang DY, Hayward JL. Plasma prolactin levels and breast cancer: relation to parity, weight and height, and age at first birth. Int J Cancer 1981;28:31–4.
52. Su X, Hankinson SE, Clevenger CV, Eliassen AH, Tworoger SS. Energy balance, early life body size, and plasma prolactin levels in postmenopausal women. Cancer Causes Control 2009;20:253–62.
53. Schernhammer ES, Tworoger SS, Eliassen AH, Missmer SA, Holly JM, Pollak MN, Hankinson SE. Body shape throughout life and correlations with IGFs and GH. Endocr Relat Cancer 2007;14:721–32.
54. Brandebourg T, Hugo E, Ben-Jonathan N. Adipocyte prolactin: regulation of release and putative functions. Diabetes Obes Metab 2007;9: 464–76.