

Immunoassay and Nb2 lymphoma bioassay prolactin levels and mammographic density in premenopausal and postmenopausal women the Nurses' Health Studies

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Abstract Higher circulating prolactin levels have been associated with higher percent mammographic density among postmenopausal women in some, but not all studies. However, few studies have examined associations with dense area and non-dense breast area breast or considered associations with prolactin Nb2 lymphoma cell bioassay levels. We conducted a cross-sectional study among 1,124 premenopausal and 890 postmenopausal women who were controls in breast cancer case-control studies nested in the Nurses' Health Study (NHS) and NHSII. Participants provided blood samples in 1989–1990 (NHS) or 1996–1999 (NHSII) and mammograms were obtained from around the time of blood draw. Multi-variable linear models were used to assess the associations between prolactin levels (measured by immunoassay or bioassay) with percent density, dense area, and non-dense area.

Among 1,124 premenopausal women, percent density, dense area, and non-dense area were not associated with prolactin immunoassay levels in multivariable models (p trends = 0.10, 0.18, and 0.69, respectively). Among 890 postmenopausal women, those with prolactin immunoassay levels in the highest versus lowest quartile had modestly, though significantly, higher percent density (difference = 3.01 percentage points, 95 % CI 0.22, 5.80) as well as lower non-dense area (p trend = 0.02). Among women with both immunoassay and bioassay levels, there were no consistent differences in the associations with percent density between bioassay and immunoassay levels. Postmenopausal women with prolactin immunoassay levels in the highest quartile had significantly higher percent density as well as lower non-dense area compared to those in the lowest quartile. Future studies should examine the underlying biologic mechanisms, particularly for non-dense area.

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Abbreviations

NHS Nurses' Health Study
BMI Body mass index
BBD Benign breast disease
CV Coefficient of variation
ICC Intraclass correlation coefficient

Introduction

Mammographic density is the radiographic appearance of the breast on a mammogram and is a strong predictor of subsequent breast cancer risk [1]. Epithelial and stromal

tissue in the female breast is radiodense and appears light on a mammogram, whereas fat is radiolucent and appears dark. Women with over 75 % dense tissue on a mammogram have 4–6 times the risk of breast cancer compared to women with little to no dense tissue [1, 2]. Mammographic density is associated with a number of reproductive factors, including parity, and is thought to represent cumulative exposure to hormones.

Prolactin, a hormone involved in the normal differentiation of mammary epithelium, may influence breast cancer risk through cellular proliferation and survival [3]. Higher circulating prolactin levels have been associated with higher mammographic density among postmenopausal women in some [4, 5], but not all studies [6, 7]. For example, in a previous analysis of 489 postmenopausal women in the Nurses' Health Study (NHS), there was no significant association between prolactin and percent mammographic density [8]. The few studies in premenopausal women have not observed an association [4, 7, 9]. In addition, only two studies examined the association with non-dense area of the breast [9, 10], with one study reporting an inverse association with prolactin levels among postmenopausal women [10]. This area is of particular interest as the dense area and non-dense area of the breast may have independent effects on breast cancer risk [11, 12].

Prior studies have determined prolactin levels using an immunoassay that measures multiple forms of circulating prolactin. However, this immunoassay may not reflect levels of bioactive prolactin, as prolactin isoforms have varying biological activities [13, 14]. The Nb2 lymphoma cell bioassay is a sensitive measure of somatolactogenic activity of prolactin in plasma [15]; however, data on prolactin bioassay levels and breast cancer or breast density are limited [16]. Therefore, the purpose of this cross-sectional study was to examine the association between circulating prolactin levels, as measured by the Nb2 lymphoma cell bioassay, with measures of mammographic density among both premenopausal and postmenopausal women in the NHS and NHSII. Further, we updated our previous analysis of prolactin immunoassay levels and percent density to include over 1,100 premenopausal women as well as approximately 400 additional postmenopausal women. Further, this updated analysis also examines the relationship between prolactin levels with dense area and non-dense area of the breast.

Materials and methods

Study population

The NHS cohort began in 1976 when 121,700 female registered nurses, 30–55 years of age and residing in 11

U.S. states completed an initial questionnaire. In 1989, 116,430 female registered nurses, aged 25–42, from 14 U.S. states completed an initial questionnaire forming the NHSII cohort. Both cohorts are followed by biennially mailed questionnaires to collect information on exposures and covariates as well as incident diseases.

In 1989–1990, 32,826 NHS participants, aged 43–70, provided blood samples. Between 1996 and 1999, we obtained blood samples from 29,611 NHSII members, aged 32–45 years. Characteristics of both cohorts and blood collection details have been described previously [17–20]. Briefly, in the NHSII, premenopausal women who had not taken any type of hormones, been pregnant, or breastfed in the previous 6 months ($n = 18,521$), provided timed blood samples on the third to fifth day of their menstrual cycle (follicular sample) and 7–9 days before the anticipated start of their next cycle (luteal sample). Follicular plasma was aliquoted by the participant between 8 and 24 h after collection and frozen. All NHS participants as well as NHSII women who were ineligible to provide timed samples (i.e., perimenopausal, postmenopausal, had a simple hysterectomy, currently used oral contraceptives or other hormones, or declined to give timed samples; $n = 11,090$) provided a single blood sample (referred to as “untimed” samples). For both timed and untimed samples, women shipped the blood to our laboratory, with an ice-pack, via overnight courier, where the samples were processed, separated into plasma, red blood cell, and white blood cell components, and aliquoted into labeled cryotubes.

We restricted our analysis to women who were controls in the NHS or NHSII nested breast cancer case–control studies and were matched to cases diagnosed between 1990 and 2004 (NHS) or 1999 and 2007 (NHSII) [17, 21]. Breast cancer cases were matched to one or two controls on age, menopausal status at blood draw and diagnosis, current postmenopausal hormone use (PMH), month, time of day, fasting status at time of blood collection, and luteal day (NHSII timed samples only). We collected mammograms conducted as close as possible to the date of blood collection for women in the nested breast cancer case–control studies. Among controls in the NHS and NHSII case–control studies, 2,939 had measures of prolactin immunoassay levels, mammographic density, and body mass index (BMI). We next excluded five controls whose prolactin immunoassay levels were determined to be outliers based on the generalized extreme studentized deviate many-outlier detection approach [22]. We further restricted our study population to the 1,124 women who were premenopausal at both blood collection and mammogram as well as 890 women who were postmenopausal at both blood collection and mammogram and who reported no current PMH use. The median time between mammography and blood collection was 9 months for premenopausal women and

13 months for postmenopausal women. As part of another nested study of prolactin Nb2 bioassay levels and breast cancer risk, 551 of these women (317 premenopausal and 234 postmenopausal) had prolactin bioassay levels measured. The study was approved by the Committee on the Use of Human Subjects in Research at the Brigham and Women's Hospital.

Laboratory assays

Prolactin was assayed by microparticle enzyme immunoassay in 12 batches for untimed samples in the NHS and in three batches for luteal, follicular, and untimed samples in the NHSII. We averaged the prolactin levels in follicular and luteal samples among women in the NHSII who provided timed samples. Samples were assayed at the Reproductive Endocrinology Unit Laboratory at the Massachusetts General Hospital using the AxSYM immunoassay system (Abbott Diagnostics, Chicago, IL) as well as by Christopher Longcope, MD (University of Massachusetts Medical Center, Worcester, MA), using the IMx System (Abbott Laboratory, Abbott Park, IL). The correlation between the two laboratories was 0.91 and across different batches within the same dataset was greater than 0.95. The limit of detection was 0.6 ng/mL. Nb2 was assayed in the NHS in 65 batches and in the NHSII in 39 batches, for a total of 104 batches consisting of approximately 25–30 women each. The Nb2 bioassay for biologically active somatotactogen was performed in duplicate as previously described [15, 23]. To assess prolactin variability, 10 % blinded replicate quality control (QC) samples were randomly interspersed across the entire set of participant samples. All immunoassay batches had QC samples; however, due to the smaller batch size of the bioassay, 77 of the 104 bioassay batches included blinded QC samples. Average within batch coefficients of variation (CVs) was less than 12 % for the immunoassay. For the bioassay, within batch CVs were calculated either using blinded QC samples ($n = 77$ batches) or using the average CV of the duplicate participant samples within a batch if blinded QCs were not available ($n = 27$ batches). For 97 of the batches, CVs ranged from 0.3 to 17.3 % (mean = 7.0 %). Seven batches had CVs above 20 % (range 21.5–26.6). Therefore, we conducted a sensitivity analysis excluding these seven batches. The between batch CV for the bioassay was 40 %.

Mammographic density

The craniocaudal views of both breasts were digitized with a Lumisys 85 laser film scanner. Using the Cumulus software for computer-assisted thresholding, we measured absolute dense area, absolute non-dense area (the total area

minus the dense area), and percent dense area (the dense area divided by the total area) and averaged the density parameters of both breasts. Images from NHS participants were read in two batches by two readers. In NHSII, a single observer read the mammograms in two batches, 3 years apart with a small number of mammograms included in both batches. While there was high reproducibility within each batch, there was evidence of between batch variability in the NHSII. Therefore, for the overall NHSII breast cancer case–control mammography dataset, we used multivariable linear regression models to estimate the effect of batch on density measurements, controlling for age, menopausal status, BMI, and case–control status [18]. We then adjusted density measurements in the second NHSII batch by adding the coefficient for mammogram batch to the raw value to estimate the measurements that would have been obtained if the mammogram had been included in the first batch. For all batches, readers were blinded to case–control status.

Covariate data

We used the covariate information from the biennial questionnaire preceding the mammogram date to obtain data on the following covariates: BMI (kg/m^2), history of benign breast disease (BBD), family history of breast cancer, age at menarche, age at first birth and parity, breastfeeding, smoking, alcohol intake, past PMH use, and age at menopause.

Statistical analysis

Due to batch-to-batch variation over time, we recalibrated prolactin immunoassay and bioassay levels from all batches to have a comparable distribution to an average batch according to methods outlined by Rosner et al. [24]. Using this method, 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 the following covariates: age, BMI, fasting status, time of blood collection, parity, history of PMH use, and use of antidepressants at the time of blood collection. Prolactin immunoassay and bioassay 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.

We used generalized linear models adjusting for matching factors to evaluate the association of prolactin immunoassay and bioassay levels with mammographic density. These models accounted for any correlation between controls that were matched to the same case. We modeled square-root-transformed dense area and non-dense area on

mammogram as outcomes since both distributions were skewed. We estimated the difference from the lowest quartile of percent density, square-root dense area, and square-root non-dense area for each quartile of prolactin levels. We tested for trend using Wald tests by ordinal modeling the median of the quartiles. Our primary model adjusted for age (continuous) and matching factors with subsequent models additionally adjusting for correlates of prolactin and breast density including history of BBD, family history of breast cancer, age at menarche, age at first birth, parity, breastfeeding, antidepressant use, smoking status, alcohol use, BMI, as well as past PMH use and age at menopause (among postmenopausal women). To determine if the associations between prolactin levels and percent density varied by age (<45, 45+ years for premenopausal; <60, 60+ for postmenopausal), BMI (<25, 25+ kg/m²), cohort (NHS, NHS2; in premenopausal women) and sample type (timed, untimed; in premenopausal women), we calculated Wald tests for the interaction term between the ordinal median variable for the hormone and the binary age, BMI, cohort, and sample type variables. In addition, we conducted a secondary analysis restricting our analysis of prolactin immunoassay levels to the 551 women who also had Nb2 bioassay levels measured to allow a better comparison between the two assays. Lastly, in sensitivity analyses, we restricted to parous women, restricted to NHS women among postmenopausal women, and excluded seven batches with CVs > 20 % in our analysis of prolactin Nb2 bioassay levels. We considered a two-sided *p* value of less than 0.05 to be statistically significant and used SAS 9.2 software (SAS Institute, Cary, NC, USA) for all analyses.

Results

For both pre- and postmenopausal women, those in the highest category of percent mammographic density were younger, had a lower BMI, were more likely to be nulliparous, and were more likely to have a history of BBD compared to women in the lowest quartile of mammographic density (Table 1).

In multivariable models, we did not observe a statistically significant association between prolactin immunoassay levels and percent mammographic density, dense area, or non-dense area in the 1,124 premenopausal women (*p* trends ≥ 0.10) (Table 2). For example, in our fully adjusted model, women in the highest quartile of prolactin levels had 2.24 % points higher percent mammographic density than women in the lowest quartile (95 % CI -0.79, 5.27, *p* trend = 0.10). Among 890 postmenopausal women, those in the highest quartile of prolactin levels had significantly higher percent mammographic density

compared to those in the lowest quartile of levels (difference = 3.01, 95 % CI 0.22–5.08); however, the trend across quartiles did not reach statistical significance (*p* trend = 0.07). Interestingly, prolactin immunoassay levels were significantly inversely associated with non-dense area (*p* trend = 0.02), but not with dense area (*p* trend = 0.40).

Next, we examined the association between prolactin and mammographic density among the 551 women with prolactin measured by both the immunoassay and Nb2 bioassay. Among 317 premenopausal women, both prolactin immunoassay and bioassay levels were positively associated with percent density, though the difference between extreme quartiles of prolactin was significant only for immunoassay levels. Women in the highest quartiles of prolactin levels had 6.51 (95 % CI 0.19, 12.82, *p* trend = 0.05) and 3.85 (-2.23, 9.93, *p* trend = 0.22) percentage point higher density compared to women in the lowest quartiles, for the immunoassay and bioassay, respectively (Table 3). The association between bioassay levels and percent density in premenopausal women was somewhat stronger when we restricted to bioassay batches with CVs < 20 % (*N* = 296; comparable difference = 5.79 % points, 95 % CI -0.56, 12.15; *p* trend = 0.08). There was no significant association between prolactin immunoassay or Nb2 bioassay levels and dense or non-dense area (*p* trends ≥ 0.09 , respectively). Among 234 postmenopausal women, there was no association between prolactin immunoassay levels and percent density, dense area, or non-dense area (*p* trend = 0.89, 0.70, and 0.79, respectively) (Table 4). In contrast, there was a significant inverse association with non-dense area (*p* trend = 0.01) as well as a non-significant positive association between prolactin Nb2 bioassay levels and percent density (*p* trend = 0.06) among these same women. Women in the highest quartile of prolactin Nb2 bioassay levels had 4.80 % points higher mammographic density compared to women in the lowest quartile (95 % CI -1.81, 11.42). However, the association between bioassay levels and percent density in postmenopausal was stronger when we restricted to the batches with CVs < 20 % (*N* = 220; comparable difference = 5.60, 95 % CI -0.96, 12.16; *p* trend = 0.03). The association between prolactin immunoassay levels and percent density did not vary by age for either premenopausal (*p*-interaction = 0.22) or postmenopausal women (*p*-interaction = 0.61) nor by BMI for either premenopausal (*p*-interaction = 0.29) or postmenopausal women (*p*-interaction = 0.45). Further, the association did not differ by cohort (*p*-interaction = 0.23) or sample type (*p*-interaction = 0.35) among premenopausal women. Lastly, the association among postmenopausal women was similar when we excluded NHS2 postmenopausal women (e.g.,

Table 1 Characteristics of 1,124 premenopausal and 890 postmenopausal women by quartile of percent mammographic density, NHS and NHSII, mean (SD) or *N* (%)

	Premenopausal				Postmenopausal			
	Percent Mammographic Density				Percent Mammographic Density			
	Q1 (<24 %) <i>N</i> = 281	Q2 (24–40 %) <i>N</i> = 281	Q3 (40–57 %) <i>N</i> = 281	Q4 (57+ %) <i>N</i> = 281	Q1 (<9 %) <i>N</i> = 222	Q2 (9–18 %) <i>N</i> = 223	Q3 (18–31 %) <i>N</i> = 221	Q4 (31+ %) <i>N</i> = 224
<i>Mean (SD)</i>								
Age (years)	47.5 (4.3)	46.8 (4.5)	45.8 (4.3)	45.4 (4.3)	62.4 (6.1)	61.9 (6.7)	61.0 (7.8)	58.1 (7.8)
BMI (kg/m ²)	29.8 (6.1)	25.6 (4.2)	24.0 (4.0)	22.2 (2.9)	30.0 (5.8)	26.9 (4.9)	25.5 (4.5)	23.5 (3.6)
Age at menarche	12.1 (1.4)	12.4 (1.4)	12.6 (1.4)	12.7 (1.4)	12.6 (1.6)	12.4 (1.3)	12.6 (1.4)	12.6 (1.5)
Parity (among parous)	2.7 (1.1)	2.6 (1.0)	2.5 (1.0)	2.3 (0.8)	3.6 (1.7)	3.7 (1.8)	3.4 (1.5)	3.0 (1.5)
Age at first birth (among parous)	25.3 (4.2)	26.1 (4.1)	26.3 (4.2)	26.2 (3.9)	25.3 (3.2)	25.0 (3.2)	25.6 (3.5)	25.7 (3.5)
Alcohol (gm/day)	4.3 (7.8)	4.1 (6.5)	5.3 (8.7)	4.9 (8.5)	5.4 (9.2)	5.3 (8.7)	4.7 (8.3)	6.6 (9.9)
Age at menopause (years)	–	–	–	–	50.0 (4.0)	49.5 (4.7)	49.2 (4.7)	47.9 (6.0)
<i>N (Percent)</i>								
Nulliparous	29 (10.3)	23 (8.2)	40 (14.2)	49 (17.4)	9 (4.1)	7 (3.1)	18 (8.1)	29 (13.0)
<i>Breastfeeding (among parous)</i>								
Never	81 (32.1)	58 (22.5)	48 (19.9)	60 (25.9)	99 (46.5)	96 (44.4)	82 (40.4)	81 (41.5)
1–18 months	100 (39.7)	119 (46.1)	105 (43.6)	114 (49.1)	96 (45.1)	96 (44.4)	99 (48.8)	91 (46.7)
18+ months	71 (28.2)	81 (31.4)	88 (36.5)	58 (25.0)	18 (8.5)	24 (11.1)	22 (10.8)	23 (11.8)
History of BBD	114 (40.6)	119 (42.4)	140 (49.8)	176 (62.6)	74 (33.3)	90 (40.4)	93 (42.1)	116 (51.8)
Family history of breast cancer	18 (6.4)	34 (12.1)	26 (9.3)	26 (9.3)	31 (14.0)	34 (15.3)	36 (16.3)	31 (13.8)
<i>Smoking</i>								
Never	158 (56.2)	158 (56.2)	184 (65.5)	189 (67.3)	95 (42.8)	95 (42.6)	126 (57.0)	120 (53.6)
Past	94 (33.5)	100 (35.6)	77 (27.4)	76 (27.1)	101 (45.5)	97 (43.5)	79 (35.8)	77 (34.4)
Current	29 (10.3)	23 (8.2)	20 (7.1)	16 (5.7)	26 (11.7)	31 (13.9)	16 (7.2)	27 (12.1)
Past PMH Use	–	–	–	–	72 (32.4)	78 (35.0)	87 (39.4)	73 (32.6)
<i>Cohort</i>								
NHS	146 (52.0)	120 (42.7)	89 (31.7)	105 (37.4)	217 (97.8)	215 (96.4)	209 (94.6)	197 (88.0)
NHSII	135 (48.0)	161 (57.3)	192 (68.3)	176 (62.6)	5 (2.3)	8 (3.6)	12 (5.4)	27 (12.1)
<i>Timing of blood sample</i>								
Untimed	199 (70.8)	180 (64.1)	139 (49.5)	151 (53.7)	222 (100.0)	223 (100.0)	221 (100.0)	224 (100.0)
Timed	82 (29.2)	101 (35.9)	142 (50.5)	130 (46.3)	–	–	–	–

difference between extreme quartiles = 3.02, 95 % CI 0.21, 5.84, *p* trend = 0.06). Overall, the observed associations were similar when we restricted to parous women (data not shown).

Discussion

In our analysis of over 1,100 premenopausal women, there was no association between prolactin immunoassay levels and mammographic density. Among 890 postmenopausal women, we observed higher percent density among women in the highest quartile of prolactin immunoassay levels as

well as an inverse association between immunoassay levels and non-dense area. In a subset of 234 postmenopausal women, there was a non-significant positive association between percent density and prolactin levels measured by the Nb2 lymphoma cell bioassay, but not for prolactin levels measured by the immunoassay. Our results are consistent with several studies that have observed no association between prolactin immunoassay levels and percent density, dense area, or non-dense area among premenopausal women [4, 7, 9]. In our previous analysis of 489 postmenopausal women in the NHS, we observed that women in the highest quartile of prolactin levels had a non-significant 3.7 % point higher percent density [25]. In our

Table 2 Differences in mammographic density measures by quartiles of plasma prolactin immunoassay levels (ng/mL) among 1,124 premenopausal women and 890 postmenopausal women in the NHS and NHSII

	Premenopausal (N = 1124)				Postmenopausal (N = 890)				p trend	p trend
	Q1 < 9.3 (N = 281)	Q2 9.3–12.5 (N = 281)	Q3 12.5–17.3 (N = 280)	Q4 > 17.3 (N = 282)	Q1 < 7.1 (N = 221)	Q2 7.1–9.4 (N = 224)	Q3 9.4–12.5 (N = 222)	Q4 12.5+ (N = 223)		
Percent density (%)										
Model 1 ^a	Ref	-0.71 (-4.07, 2.64)	3.26 (-0.14, 6.67)	3.73 (0.20, 7.27)	Ref	4.13 (1.13, 7.14)	5.99 (2.90, 9.09)	5.99 (2.76, 9.21)	<0.0001	
Model 2 ^b	Ref	0.19 (-2.66, 3.04)	2.25 (-0.66, 5.16)	2.24 (-0.79, 5.27)	Ref	2.67 (0.08, 5.26)	2.27 (-0.45, 4.98)	3.01 (0.22, 5.80)	0.07	
Square-root dense area (cm ²)										
Model 1 ^a	Ref	0.00 (-0.41, 0.41)	0.26 (-0.16, 0.67)	0.41 (-0.02, 0.85)	Ref	0.51 (0.09, 0.93)	0.41 (-0.01, 0.84)	0.65 (0.21, 1.09)	0.01	
Model 2 ^b	Ref	0.03 (-0.37, 0.42)	0.13 (-0.28, 0.53)	0.26 (-0.16, 0.68)	Ref	0.37 (-0.03, 0.76)	0.03 (-0.38, 0.44)	0.28 (-0.14, 0.70)	0.40	
Square-root non-dense area (cm ²)										
Model 1 ^a	Ref	0.02 (-0.53, 0.57)	-0.50 (-1.06, 0.05)	-0.36 (-0.94, 0.21)	Ref	-0.46 (-1.03, 0.10)	-1.08 (-1.66, -0.51)	-0.99 (-1.59, -0.40)	<0.0001	
Model 2 ^b	Ref	-0.15 (-0.56, 0.27)	-0.36 (-0.78, 0.07)	-0.12 (-0.56, 0.33)	Ref	-0.07 (-0.50, 0.36)	-0.43 (-0.87, 0.01)	-0.51 (-0.96, -0.05)	0.02	

^a Model 1: age at mammography (continuous), month of blood draw (continuous), fasting status (<8 h or unknown, ≥8 h since last meal), time of day (12 am–5:59 am, 6:00 am–7:59 am, 8:00 am–11:59 pm), and cohort

^b Includes covariates in Model 1 and history of bbd (yes/no), family history of breast cancer (yes/no), age at menarche (<12, 12, 13, 14+), parity and age at first birth (nulliparous, 1–2 children age at first birth <25 year, 1–2 children age at first birth 25–29 year, 1–2 children age at first birth ≥30 year, 3+ children age at first birth <25 year, 3+ children age at first birth 25+ year), breastfeeding (<1 month, 1–18 months, 18+ months), alcohol consumption (0, <5, 5–<15, ≥15 g/d, missing), smoking status (never, past, current), past PMH use (postmenopausal only), and age at menopause (postmenopausal only)

^c Includes covariates in Model 2 and BMI (continuous)

Table 3 Differences in mammographic density measures by quartiles of plasma prolactin immunoassay and bioassay levels (ng/mL) among 317 premenopausal women in the NHS and NHSII

	Prolactin Immunoassay (ng/ml) (N = 317)				Prolactin Bioassay (ng/ml) (N = 317)					
	Q1 < 9.2 (N = 79)	Q2 9.2–13.0 (N = 79)	Q3 13.0–17.4 (N = 79)	Q4 > 17.4 (N = 80)	p trend	Q1 < 6.4 (N = 80)	Q2 6.4–8.8 (N = 79)	Q3 8.8–11.9 (N = 78)	Q4 > 11.9 (N = 80)	p trend
Percent density (%)										
Model 1 ^a Ref	1.23 (-5.33, 7.78)	5.38 (-1.22, 11.98)	3.32 (-3.57, 10.22)	0.28	Ref	2.85 (-3.68, 9.39)	6.79 (0.28, 13.30)	5.04 (-1.70, 11.78)	0.10	
Model 2 ^b Ref	3.23 (-2.63, 9.10)	4.60 (-1.37, 10.57)	6.51 (0.19, 12.82)	0.05	Ref	3.06 (-2.80, 8.92)	3.94 (-1.95, 9.83)	3.85 (-2.23, 9.93)	0.22	
Square-root dense area (cm ²)										
Model 1 ^a Ref	0.63 (-0.19, 1.45)	0.56 (-0.27, 1.39)	0.69 (-0.17, 1.55)	0.18	Ref	-0.09 (-0.90, 0.73)	0.65 (-0.17, 1.46)	0.34 (-0.51, 1.18)	0.25	
Model 2 ^b Ref	0.64 (-0.15, 1.44)	0.23 (-0.59, 1.04)	0.90 (0.04, 1.75)	0.09	Ref	-0.14 (-0.95, 0.68)	0.43 (-0.39, 1.25)	0.34 (-0.51, 1.19)	0.28	
Square-root non-dense area (cm ²)										
Model 1 ^a Ref	0.26 (-0.78, 1.31)	-0.76 (-1.80, 0.29)	0.20 (-0.90, 1.29)	0.98	Ref	-0.59 (-1.64, 0.45)	-0.91 (-1.95, 0.13)	-0.46 (-1.54, 0.62)	0.39	
Model 2 ^b Ref	-0.03 (-0.85, 0.80)	-0.67 (-1.51, 0.17)	-0.10 (-1.00, 0.79)	0.64	Ref	-0.76 (-1.60, 0.08)	-0.46 (-1.30, 0.38)	-0.23 (-1.10, 0.64)	0.81	

^a Model 1: age at mammography (continuous), month of blood draw (continuous), fasting status (<8 h or unknown, ≥8 h since last meal), time of day (12 am–5:59 am, 6:00 am–7:59 am, 8:00 am–11:59 pm), and cohort
^b Includes covariates in Model 1 and history of bbd (yes/no), family history of breast cancer (yes/no), age at menarche (<12, 12, 13, 14+), parity and age at first birth (nulliparous, 1–2 children age at first birth <25 year, 1–2 children age at first birth 25–29 year, 3+ children age at first birth ≥25 year, 3+ children age at first birth <25 year, 3+ children age at first birth 25+ year), breastfeeding (<1 month, 1–18 months, 18+ months), alcohol consumption (0, <5, 5 ≤ 15, ≥15 g/d, missing), and smoking status (never, past, current)
^c Includes covariates in Model 2 and BMI (continuous)

Table 4 Differences in mammographic density measures by quartiles of plasma prolactin immunoassay and bioassay levels (ng/mL) among 234 postmenopausal women in the NHS and NHSII

	Prolactin Immunoassay (ng/ml) (N = 234)				Prolactin Bioassay (ng/ml) (N = 234)					
	Q1 < 7.0 (N = 60)	Q2 7.0–9.1 (N = 57)	Q3 9.1–12.5 (N = 57)	Q4 12.5+ (N = 60)	p trend	Q1 < 4.8 (N = 59)	Q2 4.8–6.6 (N = 58)	Q3 6.6–9.6 (N = 59)	Q4 9.6+ (N = 58)	p trend
Percent density (%)										
Model 1 ^a Ref	4.45 (-2.53, 11.43)	4.41 (-2.57, 11.38)	4.41 (-2.57, 11.38)	2.21 (-5.16, 9.59)	0.73	Ref	0.23 (-6.67, 7.14)	6.22 (-0.78, 13.23)	8.92 (1.80, 16.05)	<0.0001
Model 2 ^b Ref	5.07 (-1.05, 11.20)	1.45 (-4.74, 7.64)	1.07 (-5.35, 7.48)	1.07 (-5.35, 7.48)	0.89	Ref	-1.35 (-7.68, 4.98)	3.60 (-2.73, 9.94)	4.80 (-1.81, 11.42)	0.06
Square-root dense area (cm ²)										
Model 1 ^a Ref	0.37 (-0.53, 1.26)	0.14 (-0.76, 1.03)	0.47 (-0.47, 1.42)	0.47 (-0.47, 1.42)	0.42	Ref	0.01 (-0.87, 0.90)	0.21 (-0.68, 1.10)	1.07 (0.16, 1.98)	0.01
Model 2 ^b Ref	0.40 (-0.47, 1.26)	-0.09 (-0.96, 0.78)	0.30 (-0.60, 1.20)	0.30 (-0.60, 1.20)	0.70	Ref	0.02 (-0.84, 0.87)	-0.22 (-1.09, 0.64)	0.42 (-0.49, 1.33)	0.38
Square-root non-dense area (cm ²)										
Model 1 ^a Ref	-0.15 (-1.26, 0.95)	-0.77 (-1.87, 0.34)	0.06 (-1.11, 1.22)	0.06 (-1.11, 1.22)	0.96	Ref	0.36 (-0.72, 1.44)	-1.09 (-2.17, 0.00)	-1.44 (-2.55, -0.34)	<0.0001
Model 2 ^b Ref	0.04 (-0.79, 0.86)	-0.15 (-0.99, 0.68)	0.14 (-0.73, 1.00)	0.14 (-0.73, 1.00)	0.79	Ref	0.50 (-0.29, 1.30)	-0.90 (-1.69, -0.11)	-0.74 (-1.58, 0.10)	0.01

^a Model 1: age at mammography(continuous), month of blood draw (continuous), fasting status (<8 h or unknown, ≥8 h since last meal), time of day (12 am–5:59 am, 6:00 am–7:59 am, 8:00 am–11:59 pm)
^b Includes covariates in Model 1 and history of bbd (yes, no), family history of breast cancer (yes, no), age at menarche (<12, 12, 13, 14+), parity and age at first birth (nulliparous, 1–2 children age at first birth <25 year, 1–2 children age at first birth 25–29 year, 3+ children age at first birth ≥25 year, 3+ children age at first birth <25 year, 3+ children age at first birth 25+ year), breastfeeding (<1 month, 1–18 months, 18+ months), alcohol consumption (0, <5, 5 ≤ 15, ≥15 g/d, missing), smoking status (never, past, current), BMI (continuous), and past PMH use

updated analysis, the association between prolactin immunoassay levels and percent density was similar (difference of 3.0), but statistically significant due to the increased sample size. These results are consistent with two studies that observed a positive association between prolactin immunoassay levels and percent mammographic density among postmenopausal women [4, 5]. However, two other large studies ($N = 712$ and 802) reported no association [6, 7]. Differences between studies may be in part be related to the use of different assays, different sample types (e.g., serum vs plasma), and inclusion of women on PMH at blood collection and/or mammogram in some prior studies.

To our knowledge, only one study has examined the association between prolactin levels and non-dense area among postmenopausal women. Consistent with our study, McCormack et al. observed that higher prolactin levels were significantly associated with lower non-dense area, but not with dense area [10]. These findings are contrary to our hypothesis that higher prolactin levels would be associated with an increase in dense area due to the proliferative effects of prolactin on epithelial cells. Further research is necessary to confirm the potential inverse association with non-dense area as well as to understand the mechanism by which prolactin may affect the amount of fat in the breast.

There was no consistent difference in the association with percent density between prolactin immunoassay and bioassay levels. In premenopausal women, the difference between extreme quartiles was similar between the immunoassay and the bioassay after we restricted to bioassay batches with CVs below 20 %. In postmenopausal women, the association with percent density was somewhat stronger for the bioassay compared to the immunoassay, especially after excluding bioassay levels from batches with high CVs. In two small breast cancer case–control studies, the difference in prolactin levels between cases and controls was greater for the bioassay than for the immunoassay [26, 27]. While these prior studies suggest that the Nb2 lymphoma cell bioassay may measure the more biologically relevant portion of circulating prolactin, we did not observe consistent differences between the immunoassay and the bioassay with mammographic density or with breast cancer risk in the full nested case–control study [16], highlighting the need for additional research.

Our study has several limitations. While the CVs for the prolactin immunoassay are low, there is likely some measurement error. This measurement error should be non-differential as the laboratory is blinded to density measurements. Although the CVs for some bioassay batches were high, we conducted a sensitivity analysis excluding batches with higher or unknown CVs. We only have a single measurement of prolactin levels, which may not be

representative of long-term levels. However, the 3-year intraclass correlation coefficient (ICC) for the average of prolactin levels for follicular and luteal samples for both the immunoassay and bioassay was 0.64 among premenopausal NHSII women. [16, 28] Among 79 postmenopausal women in the NHS, the 3-year ICC was 0.53 for prolactin immunoassay levels [29] and among 68 postmenopausal women the NHS the ICC was 0.63 for the bioassay over 3 years [16]. These data suggest that a single measurement of prolactin is representative of levels over at least a 3-year period. Mammographic density measurements are highly reproducible though there is the potential for some random error. Since the mammogram reader was blinded to prolactin levels any measurement error should be non-differential. The strengths of our study include the centralized collection and reading of mammograms, high quality prolactin assays, and detailed adjustment for correlates of mammographic density.

Conclusion

Overall, this study suggests that prolactin levels are associated with modestly higher percent mammographic density and lower non-dense area among postmenopausal women. Additional research into the association between prolactin and non-dense area of the breast may help elucidate the role of prolactin in breast carcinogenesis.

Conflict of interest None of the authors has a competing interest to declare.

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