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Hormonal determinants of nipple aspirate fluid yield among breast cancer cases and screening controls

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Abstract

Background—Nipple aspiration fluid (NAF) utility as a biosample is limited by the variable yield across studies. We investigated the endocrine determinants of yield in an ongoing breast cancer (BC) case-control study.

Methods—118 women yielding ≥2 μL NAF and 120 non-yielders were included; serum hormones were measured; differences in median hormones were assessed using the Wilcoxon rank sum test. Odds ratios (OR) and 95% confidence intervals (95% CI) for yielder status relative to hormone levels were estimated using logistic regression, adjusting for parity and lactation, and, in premenopausal women, menstrual cycle phase (MCP).

Results—Prolactin concentrations were higher in yielders than non-yielders (premenopausal: 7.6 and 2.5 ng/mL, $p<0.01$; postmenopausal 5.3 and 2.2 ng/mL; $p<0.01$). Among premenopausal-yielders, estradiol was lower (64.3 vs. 90.5 pg/mL, MCP-adjusted $p=0.02$). In separate menopausal status and parity-adjusted models, significant case-control differences persisted in prolactin: case OR 1.93 (95% CI 1.35, 2.77), control OR 1.64 (95% CI 1.17, 2.29). Premenopausal control yielders had higher progesterone (OR 1.70, 95% CI 1.18, 2.46) and sex-hormone binding-

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globulin (OR 2.09, 95% CI 1.08, 4.05) than non-yielders. Among parous women, further adjustment for lactation suggested a stronger positive association of serum prolactin with yield in cases than controls.

Conclusion—NAF-yielders show higher prolactin than non-yielders, regardless of menopause and parity; implications of this and other endocrine differences on NAF biomarkers of breast cancer risk deserve further study.

Impact—NAF yield is associated with a distinct endocrine environment which must be considered in studies of NAF-based breast cancer risk markers.

Introduction

Progress in breast cancer prevention requires accurate identification of high-risk women. The exposure of breast parenchyma to a variety of hormones contributes to breast cancer risk. There is increasing realization that the local hormonal environment in the breast can differ from that in serum, and that the levels of estradiol, its precursors, and progesterone, are higher in nipple aspiration fluid (NAF) than in serum (1). NAF is obtained by applying suction to the non-lactating breast, and was identified as a potential biosample by Papanicolaou in 1958 (2). Initial interest focused on cytologic features of cells in NAF and their use for early diagnosis (3) or risk stratification in asymptomatic women (4, 5). In recent years, there is increasing interest in the protein and hormone constituents of NAF (6, 7)(8), with the idea that NAF provides a window of observation into the local protein and endocrine environment of the breast. We previously showed strong correlations between NAF hormone and NAF protein constituents, but not between serum hormone levels and NAF proteins (9). These findings strengthen the argument that local breast levels of estrogenic steroids are biologically more relevant than circulating levels.

We are conducting a case-control study to assess the relationships of NAF sex steroid concentrations with breast cancer risk, to test the hypothesis that higher sex steroid levels in NAF increase risk. NAF is the simplest, best tolerated, and the least expensive of the available breast sampling techniques. Its disadvantage relates to the variable fraction of NAF-yielders, ranging from 30% to 90% in various studies, depending, in part, on the volume of NAF considered an adequate sample (6, 10). This variability has important implications for NAF-based research. Biomarkers discovered in NAF cannot be measured in non-yielders, and it is possible that systemic factors related to NAF yield may also be related to secretory activity of the breast and breast cancer risk, so that non-yielders are distinct from yielders. Several studies have identified age, race, and reproductive history as determinants of NAF yield (11), but the systemic endocrine profiles of yielder and non-yielder populations have not been studied. Finally, data suggesting that NAF yield in and of itself is a breast cancer risk factor (12) provide further impetus to understand its determinants. The NAF-yield rate in our on-going study averages 60%, providing the opportunity to analyze hormone levels, and percent mammographic density (MD) in yielders and non-yielders.

Materials and Methods

This study was approved by the Northwestern University Institutional Review Board; participants aged 30–70 years provided informed consent, and completed a questionnaire regarding demographic, reproductive, breast health and family history. Cases were women presenting to the Lynn Sage Breast Center with newly diagnosed unilateral breast cancer, and controls were women presenting for routine screening, with a normal breast examination and mammogram. Women using oral contraceptives or postmenopausal hormones during the prior 3 months were excluded. A subset of 238 participants was randomly selected from the parent case-control study. These included equal numbers of yielders and non-yielders, cases and controls, pre- and postmenopausal, and frequency matched for age and race.

Menopause was defined as >12 months without a menstrual period in women with intact ovaries, with serum FSH of >30 mIU/mL and serum estradiol of <30 pg/mL. The menstrual cycle phase (MCP) was designated as follicular, mid-cycle or luteal, counting backwards from the date of the next menstrual period (NMP) to the last menstrual period (LMP), with NMP=day 0. Luteal phase was 0 to −12 days; midcycle was day −13 to −21; follicular phase was days −22 to LMP. Serum hormonal criteria were: follicular phase estradiol <60 pg/mL, P4<3.0 ng/mL; midcycle estradiol>60 pg/mL, P4 <3.0 ng/mL; luteal phase: estradiol >30 pg/mL, P4 >3.0 ng/mL. Self-reported postmenopausal status was reassigned to premenopausal in six hysterectomized women with an intact ovary, based on hormonal criteria. Three women with FSH > 30 mIU/mL and estradiol <30 pg/mL were switched from pre- to post-menopausal. This resulted in a total of 238 women (122 pre- and 116 post-menopausal) included in analysis.

For NAF collection the breast was warmed for 20–30 minutes, massaged for 5 min, and an aspirator (Cytyc Corporation, Marlboro MA) applied, along with manual stripping of the nipple to obtain fluid. The fluid was collected in a calibrated capillary tube. In women with breast cancer, only the unaffected breast was sampled. Women yielding ≥2 μL NAF were designated as yielders since this is the minimal volume of NAF required for biomarker assays; all others were designated non-yielders. The volume of NAF collected depended on a number of factors, including the willingness of the subject to undergo continued collection.

Blood was drawn at the same time for assay of serum estradiol, progesterone, follicle stimulating hormone (FSH), and sex hormone binding globulin (SHBG). Collection time for both NAF and blood ranged from 9 am to 3 pm; morning and afternoon times were balanced between cases and controls. Serum prolactin was measured using the NB2 assay for biologically active somatolactogen, as previously described (13). Estradiol and progesterone were assayed with radioimmunoassay kits from Beckman Coulter. The sensitivity is 2.2 pg/mL; 17β-estradiol 3-glucuronide cross-reacts 2.56%; all other steroids cross-react <1%. The sensitivity of the progesterone assay is 0.1 ng/mL; among naturally occurring steroids, deoxycorticosterone cross-reacts 1.7%; all others cross-react <1.0%. FSH and SHBG were assayed with a sandwich-type enzyme immunoassay kit from Alpco Diagnostics. The sensitivity of the FSH assay is 1.0 mIU/mL; cross-reactivity with human chorionic gonadotropin (hCG) and human luteinizing hormone (hLH) was not detectable and with hTSH was <4 mIU/mL. The sensitivity of the SHBG assay is 0.1 nmol/L; cross-reaction

with thyroxine-binding globulin was not detectable. The percent intra- and interassay coefficients of variation were; estradiol 10.4 and 17.2; progesterone 2.6 and 8.1; FSH 7.2 and 7.6; SHBG 8.5 and 14.4% respectively. Unconjugated estradiol and non-SHBG-bound estradiol (non-SHBG-estradiol) were estimated from the concentrations of total estradiol and its serum binding proteins (14). Free estradiol concentrations [F] were calculated from $F^2 \times a + F \times b - c = 0$ by the quadratic equation where $a = Ks + KaKs[A]$, $b = 1 + Ka[A] + Ks[S] - Ks[E]$, and $c = [E]$. "E" is the measured serum estradiol concentration in each subject in nM and "S" is the measured serum SHBG concentration in nM. The constants were set as follows: serum albumin concentration, $[A] = 7 \times 10^4$ nM; binding affinity of estradiol for SHBG, $Ks = 17\ nM^{-1}$; binding affinity of estradiol for albumin, $Ka = 3 \times 10^{-5}\ nM^{-1}$.

The most recent mammographic image within 4 months of the study visit was obtained. Mammographic density (MD) was quantified using CUMULUS® software on digital and digitized images (15). The craniocaudal view was used for analysis, and the breast density measurement matched the breast from which NAF was collected. The images were analyzed by a single reader, with 20% of the images also analyzed by a second reader. The Spearman Correlation between the two readers was 0.83.

Statistical methods

Initial power calculations assuming 90% power to detect 1 standard deviation at 5% 2-sided type 1 error indicated a sample size of 40 subjects per group (premenopausal cases, premenopausal controls, postmenopausal cases, and postmenopausal controls); sample size was increased to include an additional 20 women per group to accommodate statistical control for MCP. Each of the four groups of 60 women had approximately 30 NAF yielders and 30 non-yielders.

The associations between NAF yield and patient characteristics were tested by menopausal status and then case-control status. Age and age at menarche were tested using t-tests. Race,, body mass index (BMI), any previous breast biopsy (yes/no), family history (yes/no), parity (yes/no), number of births, years from last birth, lactation (yes/no), duration of lactation and menstrual cycle phase were examined stratified by menopausal status and then further stratified by case-control status. Wilcoxon Rank-Sum Tests were used for continuous and Fisher's Exact Tests for categorical variables. Parameters related to parity are only available for parous women.

We assessed differences in the distribution of serum hormone values and %MD for yielders vs. non-yielders separately for cases and controls and menopausal groups using Wilcoxon Tests. Multivariate logistic regression was used to further test the relationship between yielder status and %MD and each log-transformed serum hormone covariate, adjusting for MCP in premenopausal women. Lastly additional multivariate models evaluated %MD and the log-transformed serum hormone covariates; this included adjustment for menopausal status (in the entire population), MCP (in premenopausal women), and for parity-related variables in parous women. In addition, we created a variable combining parity and lactation status (nulliparous women, parous women who did not lactate, parous women with lactation history < 1 year and parous women with lactation history > 1 year), so that we could adjust for lactation and still include the entire population rather than just parous women. The odds

ratios (ORs) are presented per unit increase in the log-transformed serum hormone concentration. All analyses were performed using SAS 9.3.

Results

In 238 women, we assessed the relationship between subject characteristics and NAF yield stratified by menopausal status (Table 1). There were no significant differences between yielders and non-yielders with regard to reproductive factors with a few exceptions. Among postmenopausal women, yielders were younger at menarche (12.2 vs. 12.7 years, p=0.04) and had a marginally shorter interval between enrollment and last birth (26 vs. 29 years, p=0.08) than non-yielders. In both pre- and postmenopausal women, parity, number of births, history of lactation, and duration of lactation did not differ by yielder status. For premenopausal women, yielder status was not associated with menstrual phase.

We examined the association between serum hormones and NAF yield stratified by menopausal status; data from premenopausal women were re-examined adjusting for MCP (Table 2). Median serum prolactin concentrations were significantly lower in non-yielders than yielders for both premenopausal and postmenopausal women, and among premenopausal women, following MCP adjustment. Median serum estradiol was significantly lower in yielding than in non-yielding premenopausal subjects, as was calculated free estradiol ($p < 0.01$); this finding persisted with MCP adjustment. Serum progesterone concentrations were higher in premenopausal yielders than non-yielders (p=0.04), but this difference was attenuated following MCP adjustment (p=0.08). Premenopausal yielders also had higher SHBG than non-yielders (p=0.03), but again this association was attenuated following MCP adjustment (p=0.08). There were no significant differences by yielder status in serum FSH or mammographic density.

Since NAF yield has been proposed to be a marker of increased breast cancer risk (12), we performed exploratory analyses of yielder status further stratified by case-control status (Table 3). We observed similar patterns for serum prolactin levels as in the combined case-control population, with significantly lower prolactin levels in non-yielding premenopausal and postmenopausal women, both cases and controls (p=0.01 in all four categories, see Table 3). However, the lower estradiol levels seen in premenopausal NAF yielders remained significant only in cases, p=0.05. The control premenopausal yielders also had a lower median total estradiol concentration, but this was not significant (p=0.37). There were no differences in the serum concentrations of progesterone, FSH, SHBG, or mammographic density in the case-controls subsets. Other parameters examined (BMI, parity, lactation, number or recency of births, or duration of lactation) were similar between yielders and non-yielders, regardless of case-control status (data not shown). Age of menarche was significantly different between yielders and non-yielders only for postmenopausal controls (12.0 and 12.9 years, p=0.04).

Since parity and a history of lactation have been reported to be determinants of NAF yield (11), we performed analyses adjusted for these parameters using logistic regression models (Table 4). Among all women, following adjustment for menopausal status and parity, the odds of NAF yield remained positively related to serum prolactin (OR 1.75, 95% CI 1.37,

2.23) and to serum progesterone (OR 1.35, 95% CI 1.08, 1.69), but not to serum free or total estradiol or to serum SHBG. Among cases, only serum prolactin was significantly related to NAF yield (OR 1.93, 95% CI 1.35, 2.77). Among controls serum prolactin remained significantly associated with NAF yield (OR 1.64, 95% CI 1.17, 2.29), but in addition, significant associations were seen for serum progesterone (OR 1.70, 95% CI 1.18, 2.46) and serum SHBG (OR 2.09, 95% CI 1.08, 4.05). Next, we assessed the combined effect of parity and lactation, using a single variable, with ascending value for parity and for parity with short or long lactation (see methods). Multivariate logistic models were adjusted for menopausal status and this combined pregnancy-lactation variable, (see Table 4). The addition of lactation made no difference to the effects seen in the parity-adjusted models; odds ratios were essentially identical and significant associations were observed for the same hormones (prolactin in all subsets, progesterone and SHBG in controls only). Estradiol, FSH, and mammographic density were not significantly different between yielders and non-yielders in the multivariate models.

Since differences in serum prolactin were seen consistently between yielders and non-yielders in all analyses, we examined prolactin patterns related to pregnancy and lactation in more detail, adjusting for parity and lactation separately, and looking for case-control differences in the prolactin relation to yielder status, as shown in Table 5. The findings were consistent with those described above. Among cases, adjustment for menopause, parity and lactation (parity yes or no, number of births, recency of last birth, lactation yes or no, and duration of lactation), did not affect the significant association of serum prolactin levels with NAF yield; odds ratios ranged from 1.81 to 1.93. Among controls, the association of NAF yield with serum prolactin values was similar to that in cases when adjusted for parity and number of pregnancies, but non-significant when adjusted for recency of parity and lactation, with ORs of 1.29 and 1.28.

Finally, there were no significant relationships between serum prolactin and any of the categorical parity variables (any parity, any lactation, and combinations of parity and lactation, $p>0.17$ for all). Similarly, there were no significant correlations between log-transformed serum prolactin and the continuous parity variables (years since last birth, number of term births, and months of lactation) with the largest correlation coefficient being −0.14.

Discussion

Determinants of NAF yield, particularly as they relate to breast cancer risk, need to be better understood in order to interpret the significance of NAF-based biomarkers. The systemic endocrine environment is a logical determinant of NAF yielder status, but has not been examined to date. We have studied 238 randomly selected participants from an on-going breast cancer case-control study, balanced for age and race, and powered to assess associations of NAF yield with serum hormone concentrations. We found that serum concentrations of prolactin were significantly lower in non-yielders of NAF in all subsets examined (pre- and postmenopausal, case and control). This association was significant in unadjusted analyses in both cases and controls, and persisted following adjustment for a combined parity-lactation variable (Table 4). Previously reported associations of serum

prolactin with parity-Fought, Angela 12 related variables suggest a hypothesis that risk increases when prolactin is not strongly suppressed after birth; we did not observe any significant relationship of serum prolactin or NAF yield with any aspect of parity or lactation.

Importantly, serum prolactin values in our NAF yielders are in the same range as other published studies. One study reported a range from 7.2 ng/mL in premenopausal women to 5.4 ng/mL in postmenopausal women (16). In a second study, the unadjusted geometric mean (interquartile range) prolactin concentrations were 10.89 ng/mL (7.80 to 15.30) in premenopausal and 6.99 ng/ mL (IQ range 5.30 to 8.60) in postmenopausal women (17). We did not use the geometric mean in our analyses, but the corresponding values for premenopausal women were 6.12 ng/mL and for postmenopausal women were 4.13 ng/mL. We observed low serum prolactin levels in association with non-yield of NAF in both cases and controls, regardless of parity and lactation when all women were included in the model (Table 4). However, among parous women (Table 5), *cases* demonstrated significantly and consistently increased ORs for NAF yield with higher serum prolactin in all models. Adjustments for parity, number of births, recency of last birth, lactation, and recency of lactation did not substantially affect the positive association of serum prolactin with NAF yield, with ORs ranging from 1.81 to 1.93. Among *controls* on the other hand, although ORs were similar to those in cases in models including parity, among parous controls the addition of recent parity or lactation resulted in lower and non-significant ORs; this could be related to fewer parous controls than cases (59 vs. 92), but the smaller point estimates (ORs ranging from 1.29 to 1.65), provide a hint of a differential effect between cases and controls.

These findings suggest that in a subset of controls (those recently parous and those who have lactated) NAF production is not determined by high serum prolactin levels. Thus *if* NAF yield driven by high serum prolactin defines a high risk state, this would not apply to recently parous women or those who have lactated. Overall we did not observe robust case-controls differences in the high prolactin-NAF yield association; however, our findings generate a hypothesis that women whose serum prolactin is successfully suppressed by parity and lactation are NAF non-yielders. The resultant breast cancer risk implications should be pursued in future, larger studies.

Our findings are of particular interest since prolactin has received increasing attention as a promoter of breast cancer. Epidemiological investigations have shown that higher serum prolactin levels are related to increased breast cancer risk(18), that the protective effect of parity may be mediated through a permanent lowering of serum prolactin following pregnancy (18), and higher circulating levels of prolactin precede the diagnosis of breast cancer (19). Prolactin contributes to breast tumorigenesis both at endocrine and autocrine/ paracrine levels through the Jak2/Stat3/Stat5 pathways (20). On the biological level, details of this pathway continue to be elaborated, but epidemiologic data supporting the contribution of prolactin to breast cancer risk point to a 2-fold risk increase when comparing women in the highest quartile of serum prolactin to the lowest quartile (21).

We observed no consistent relationships of NAF yield to the other hormones. Among premenopausal subjects, serum estradiol concentrations were significantly lower in NAF

yielders; after case-control stratification this remained significant only in cases, although the direction and magnitude of the difference was similar in premenopausal controls. The results were similar and stronger for calculated free estradiol. This suggests that NAF yield resembles the lactational state since soon after birth, estrogen levels drop while prolactin remains elevated, a phenomenon that is thought to be essential for promoting lactation. Thus low serum estradiol and elevated prolactin levels are associated with lactation whereas non-lactating women have a higher ratio of estradiol to prolactin (22). We also observed a trend towards higher progesterone levels in premenopausal NAF yielders, which persisted following adjustment for menstrual phase, and was observed in controls in parity-adjusted logistic regression models; this deserves to be pursued in future studies. A similar finding for SHBG is difficult to explain; we measured this protein mainly to allow examination of free vs. bound estradiol. The possibility of a case-control difference in this analysis suggests that SHBG should be retained as a protein of interest in future studies. Overall, therefore, it appears that serum prolactin is a consistent and strong determinant of NAF yield in all women, and among cases it is the dominant determinant; among controls, other hormones (estradiol, progesterone, SHBG) come into play, with variable results in different analytic models. These potential cases-control differences clearly need further pursuit.

Since the hormonal fluctuation of the menstrual cycle is complex and involves hormones other than those we measured, we performed analyses adjusted for menstrual cycle phase. We divided MCP into three phases, based on robust parameters (last and the next menstrual period dates, serum estradiol and progesterone). The three phases capture the major fluctuations that are observed in cycling women (low estradiol and progesterone in follicular phase, the estradiol peak of mid-cycle, and the high progesterone with moderately high estradiol in luteal phase). We feel that this is more meaningful than adjustment to day of cycle, since hormonal changes through the cycle are not linear. Although it possible that we have not fully accounted for menstrual cycle variation, we find no hint of a relation of MCP to NAF yield or volume. Not surprisingly, the positive relationship of serum progesterone to NAF yield in premenopausal women is attenuated when we adjust for MCP. Notably, the relation of NAF yield to serum prolactin concentration is far stronger and more robust than to estradiol concentration, and serum prolactin does not display significant variation with the menstrual cycle(23) (24).

It is unlikely that the nipple aspiration procedure, which was variably performed either before or after the blood sample was taken (we did not record the sequence) would have influenced serum prolactin levels. The connection to breast stimulation and hormonal release is more likely to occur with oxytocin rather than prolactin. In a study where serum prolactin levels were measured prior to and following mammography and ultrasonography of the breast, there was no significant difference in these values (16). Further, we did not find significant effects of parity or lactation on NAF yield, probably related to the fact that we designed the study to have balanced numbers of pre and postmenopausal women, yielders and non-yielders.

The definition of NAF yield varies depending of the purpose of specific studies. For example, when NAF was being elicited in order to identify the location of a ductal orifice for cannulation for lavage, any glimmer of fluid was considered positive (25). For biomarker

studies, higher thresholds have been used, but are rarely defined. We chose the 2μL cut-off based on the minimum volume required for assay performance, as determined in prior studies of NAF hormone assays. It is possible that high yielders differ from low yielders; we will be able to explore this in the final analysis of our case-control study.

There has been some discussion in the literature that NAF yielders may be at higher risk of developing breast cancer than non-yielders (12), but this observation has not been replicated. Although our data on serum prolactin and yield may support this concept, the large variation in NAF yield in different studies (6, 10), and the potential contribution of NAF collection technique to the designation of yielder or non-yielder, renders it unlikely that NAF yield in and of itself will be included in risk prediction models in the foreseeable future. What is more likely is that biomarkers discovered in NAF may be extendable to other samples that can be obtained in all women (serum, urine, saliva, breast biopsy or cytology samples), and that such universally applicable biomarkers may be included in models for assessing breast cancer risk. Thus NAF is probably most useful as a discovery platform for biomarker identification, lending urgency to the need to understand yielder-non-yielder differences.

In summary, our findings suggest that NAF yield defines an endocrine environment characterized by increased serum prolactin levels in pre- and postmenopausal cases and controls, regardless of parity history, with the possible exception of controls who were recently parous and those who lactated. Furthermore, lower estradiol levels in premenopausal NAF yielders suggest features resembling a lactational state; trends in serum progesterone and SHBG in premenopausal women deserve further investigation. These results generate new hypotheses regarding the relationship of pregnancy and lactation to the suppression of serum prolactin, NAF yield, and breast cancer risk that require testing in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Assessing the relationship between yielder status and the patient characteristics stratified by menopausal status.

	Premenopausal			Postmenopausal		
Characteristic	**Yielder (n=62)**	**Non-yielder (n=60)**	**P**	**Yielder (n=56)**	**Non-yielder (n=60)**	**P**
Cases	32	30		27	30	
Age° Mean (SD)	45.5 (3.8)	46.4 (3.7)	0.17	57.5 (5.7)	58.8 (5.8)	0.22
Race			0.73			0.51
African American	12 (19)	8 (13)		14 (25)	13 (22)	
Caucasian	44 (71)	45 (75)		39 (70)	46 (77)	
Other	6 (10)	7 (12)		3 (5)	1 (2)	
Menarche Age° Mean (SD)	12.8 (1.4)	12.9 (1.8)	0.81	12.2 (1.3)	12.7 (1.5)	0.04
BMI (kg/m2)	26 (23–31)	27 (23–32)	0.23	28 (25–35)	28 (23–32)	0.38
Any previous breast biopsy	7 (11)	5 (8)	0.76	6 (11)	8 (13)	0.78
Positive family history	8 (13)	4 (7)	0.36	5 (9)	6 (10)	0.99
Parous	38 (61)	34 (57)	0.71	42 (75)	37 (62)	0.16
Number Births	2 (0–3)	1(0–2)	0.15	2 (0.5–2)	1.5 (0–2)	0.50
Years since last birth	11 (7–16)	12 (8–19)	0.49	26 (19–31)	29 (23–34)	0.08
Ever Lactated	32 (84)	28 (82)	0.99	25 (60)	23 (62)	0.82
Duration of lactation (Months)	9 (3–23)	7.5 (1.5–17)	0.57	5.5 (0–16)	1.5 (0–13)	0.80
Menstrual Cycle Phase			0.48			NA
Follicular	15 (25)	10 (19)		NA	NA	
Mid-cycle	28 (47)	31 (58)		NA	NA	
Luteal	17 (28)	12 (23)		NA	NA	

Notes: The values are medians (25%–75% percentiles) for continuous variables, except age and age at menarche (mean (SD)), and frequency and percent for categorical variables.

° t-Tests were used for age and age at menarche, while Wilcoxon Tests were used for the remaining continuous variables. Fisher's Exact Tests were used for categorical variables.

Table 2

Yielder status and the relationship between serum hormones and mammographic density stratified by menopausal status.

	Postmenopausal				Postmenopausal		
Characteristic	**Yielder n=62 median (I-Q range)**	**Non-yielder n=60 median (I-Q range)**	**p*a***	**p*b***	**Yielder n=56 median (I-Q range)**	**Non-yielder n=60 median (I-Q range)**	**p*a***
Prolactin (ng/mL)	7.6 (4.5–17.1)	2.5 (1.6–8.1)	<0.01	<0.01	5.3 (3.4–10.7)	2.2 (1.3–4.2)	<0.01
Total estradiol (pg/mL)	64.3 (40.2–116.1)	90.5 (59.0–140.1)	0.03	0.02	17.1 (10.6–23.8)	21.2 (12.5–29.5)	0.23
Free estradiol (pg/mL)	0.07 (0.04–0.12)	0.10 (0.07–0.19)	<0.01	0.01	0.02 (0.01–0.04)	0.02 (0.01–0.05)	0.45
Progesterone (ng/ml)	1.7 (0.6–4.2)	0.8 (0.3–2.8)	0.04	0.08	0.3 (0.2–0.5)	0.3 (0.1–0.4)	0.44
FSH (mIU/mL)	6.9 (3.5–11.5)	5.7 (3.2–18.1)	0.79	0.68	69.9 (60.0–93.1)	70.5 (58.3–85.5)	0.72
SHBG (nmol/L)	1061 (767–1516)	900 (633–1259)	0.03	0.08	776 (567–1395)	856 (600–1166)	0.68
Mammographic Density(%)	21.4 (8.5–27.0)	20.5 (13.0–30.5)	0.44	NA	10.7 (6.1–16.7)	12.8 (4.4–22.4)	0.24

Notes: FSH: Follicle Stimulating Hormone; SHBG: Sex Hormone Binding Globulin

[a] Wilcoxon Tests using raw serum hormone values and % dense area on mammogram were used to test differences between yielders and non-yielders stratified by menopausal status.

[b] For premenopausal women, logistic regression analyses was performed to adjust for menstrual cycle phase at the time of NAF collection, using log transformed hormone values.

Table 3

Yielder status in relation to serum hormones and mammographic density stratified by menopausal and casecontrol status.

Cases						
	Premenopausal			Postmenopausal		
Parameter Median (I-Q range)	Yielder n=32	Non-yielder n=30	P*	Yielder n=27	Non-yielder n=30	P*
Prolactin (ng/mL)	6.8 (4.6–15.7)	3.2 (1.8–8.8)	**0.01**	5.4 (3.3–13.4)	2.1 (1.3–4.2)	**0.01**
Total estradiol (pg/mL)	65.0 (28.3–109.8)	85.3 (62.7–137.2)	**0.05**	17.5 (13.3–28.8)	23.1 (15.1–31.3)	0.34
Free estradiol (pg/mL)	0.06 (0.03–0.10)	0.11 (0.07–0.17)	**0.01**	0.03 (0.02–0.06)	0.03 (0.02–0.04)	0.48
Progesterone(ng/mL)	1.1 (0.4–3.1)	0.8 (0.2–2.3)	0.22	0.2 (0.1–0.3)	0.3 (0.1–0.4)	0.54
FSH (mIU/mL)	7.3 (4.0–13.7)	6.8 (4.6–19.9)	0.77	67.6 (52.3–91.7)	67.3 (53.5–77.8)	0.61
SHBG(nmol/L)	1022 (857–1381)	955 (733–1348)	0.32	618 (435–1066)	864 (596–1094)	0.17
Mammographic Density (%)	24.2 (12.6–30.1)	27.0 (17.5–33.1)	0.23	11.0 (6.6–17.0)	17.5 (7.3–27.5)	0.10
Controls						
	Premenopausal			Postmenopausal		
Characteristic	Yielder n=30	Non-yielder n=30	P	Yielder n=29	Non-yielder n=30	P
Prolactin(ng/mL)	7.9 (4.3–17.1)	2.5 (1.5–6.9)	**0.01**	5.1 (3.6–9.2)	2.4 (1.3–3.9)	**<0.01**
Total estradiol (pg/mL)	64.3 (53.5–120.9)	93.9 (51.9–147.0)	0.37	14.6 (8.3–19.5)	17.3 (9.2–28.3)	0.45
Free estradiol (pg/mL)	0.07 (0.04–0.17)	0.09 (0.07–0.20)	0.08	0.01 (0.01–0.02)	0.02 (0.01–0.05)	0.13
Progesterone (ng/mL)	2.2 (1.1–4.5)	0.8 (0.4–3.3)	0.09	0.4 (0.2–0.6)	0.2 (0.1–0.4)	0.08
FSH (mIU/mL)	5.1 (3.1–9.7)	5.2 (3.0–7.5)	0.97	70.5 (62.4–95.2)	76.7 (62.2–89.1)	0.85
SHBG(nmol/L)	1107 (706–1712)	785 (534–1153)	0.08	1108 (770–1449)	854 (604–1232)	0.12
Mammographic Density (%)	15.8 (7.0–25.1)	16.7 (10.9–23.3)	0.99	10.2 (5.0–15.6)	10.3 (3.5–18.9)	0.89

*Wilcoxon rank-sum test

Table 4

The relationship between NAF yield and the hormones and mammographic density, assessed using logistic regression adjusted for menopausal status, parity and lactation*. **Significant odds ratios are bolded.**

Covariate	Adjusted for menopause and parity			Adjusted for menopause, parity and lactation**		
	All n=238 OR (95% CI)	Cases n=119 OR (95% CI)	Controls n=119 OR (95% CI)	All n=238 OR (95% CI)	Cases n=119 OR (95% CI)	Controls n=119 OR (95% CI)
Prolactin	**1.75 (1.37, 2.23)**	**1.93 (1.35, 2.77)**	**1.64 (1.17, 2.29)**	**1.76 (1.38,2.24)**	**1.97 (1.37, 2.85)**	**1.85 (1.28, 2.68)**
Total Estradiol	0.81 (0.58, 1.12)	0.74 (0.46, 1.20)	0.89 (0.55, 1.42)	0.80 (0.57, 1.11)	0.75 (0.46, 1.23)	0.88 (0.54, 1.42)
Progesterone	**1.35 (1.08, 1.69)**	1.14 (0.85, 1.54)	**1.70 (1.18, 2.46)**	**1.35 (1.08, 1.70)**	1.21 (0.88, 1.65)	**1.79 (1.22, 2.61)**
FSH	0.92 (0.65, 1.30)	0.97 (0.58, 1.64)	0.89 (0.55,1.43)	0.93 (0.66, 1.31)	0.95 (0.56, 1.60)	0.92 (0.56, 1.49)
SHBG	1.44 (0.90, 2.31)	0.85 (0.40, 1.83)	**2.09 (1.08, 4.05)**	1.44 (0.89, 2.31)	0.90 (0.42,1.93)	**2.04 (1.05, 3.98)**
Mammographic Density (%)	0.98 (0.96, 1.01)	0.97 (0.94, 1.00)	1.00 (0.97, 1.03)	0.98 (0.96, 1.01)	0.97 (0.94, 1.00)	1.00 (0.97, 1.03)

* Odds ratios are presented for each unit increase in log-transformed hormone values.

** Nulliparous, parous no lactation, parous-lactation<1year, parous-lactation≥1 year.

Table 5

Serum prolactin relative to parity and lactation in cases and controls : multivariate logistic regression models, adjusted for menopause and a reproductive variable*. **Non-significant odds ratios are bolded.**

	All subjects n=238 Parous n=151		Cases n=119 Parous n=92		Controls n=119 Parous n=59	
Adjusted for menopausal status and:	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**
Parity (yes/no)	1.75 (1.37, 2.23)	<0.01	1.93 (1.35, 2.77)	<0.01	1.64 (1.17, 2.29)	<0.01
Number of births	1.72 (1.35, 2.19)	<0.01	1.81 (1.28, 2.55)	<0.01	1.65 (1.18, 2.32)	<0.01
Among parous women only						
Recency of last birth	1.57 (1.19, 2.09)	<0.01	1.86 (1.25, 2.78)	<0.01	**1.29 (0.85, 1.94)**	**0.23**
Lactation (yes/no)	1.59 (1.20, 2.12)	<0.01	1.88 (1.25, 2.83)	<0.01	**1.28 (0.84, 1.94)**	**0.25**
Months of lactation	1.59 (1.19, 2.11)	<0.01	1.91 (1.26, 2.89)	<0.01	**1.29 (0.86, 1.95)**	**0.22**

*Odds ratios are presented for each unit increase in log-transformed serum prolactin