Published in final edited form as:

Horm Cancer. 2016 April ; 7(2): 127–136. doi:10.1007/s12672-016-0252-7.

Nipple aspirate fluid hormone concentrations and breast cancer risk

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Abstract

Prior reports identify higher serum concentrations of estrogens and androgens as risk factors for breast cancer, but steroids in nipple aspirate fluid (NAF) may be more related to risk.

Incident breast cancer cases and mammography controls were recruited. Sex steroids were measured in NAF from the unaffected breasts of cases, and one breast of controls. Menopausal status and menstrual cycle phase were determined. NAF steroids were purified by HPLC and quantified by immunoassays. Conditional logistic regression models were used to examine associations between NAF hormones and case-control status. NAF samples from 160 cases and 157 controls were evaluable for hormones. Except for progesterone and DHEA, the NAF and serum concentrations were not significantly correlated. NAF estradiol and estrone were not different between cases and controls. Higher NAF (but not serum) DHEA concentrations were associated with cases, particularly among estrogen receptor (ER)-positive cases (NAF OR = 1.18, 95% CI 1.02, 1.36). NAF DHEA was highly correlated with NAF estradiol and estrone but not with androstenedione or testosterone. Higher progesterone concentrations in both NAF and serum were associated with lower risk of ER-negative cancer, NAF OR=0.69 95% CI 0.51, 0.92).

However, this finding may be explained by case-control imbalance in the number of luteal phase subjects (2 cases and 19 controls). The significantly higher concentration of DHEA in NAF of cases and its correlation with NAF estradiol indicates a potentially important role of this steroid in breast cancer risk; however, the negative association of progesterone with risk is tentative.

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INTRODUCTION

The importance of estradiol in the promotion of breast cancer is evidenced by the chemopreventive and therapeutic success of anti-estrogens and aromatase inhibitors [1, 2]. However, the association of serum estradiol with breast cancer risk [3] in postmenopausal women [4–6] and possibly in premenopausal women [7, 8], is relatively weak, perhaps because serum and tissue concentrations of estradiol are poorly correlated [9, 10]. For estradiol, several studies report correlations of 0.37 or less [11–13], although a higher correlation has been reported recently in BRCA1/2 carriers [14]. Overall the results indicate that local formation of estradiol rather than simple diffusion from blood is the primary source of estradiol in the breasts of both pre- and postmenopausal women. Androgens, which may serve as estrogen precursors or as risk factors themselves, have also been associated with higher risk of breast cancer in both pre- and postmenopausal women [15–20], particularly in hormone receptor positive breast cancer [18]. Estrogens can be synthesized in the breast from androgens of adrenal origin that diffuse into the breast [20] or from circulating estrogen sulfates [21] that are transported into the parenchymal tissue [22] and hydrolyzed by active sulfatase enzymes [11]. Regardless of whether hormones in nipple fluid represent local synthesis, or delivery through the circulation, the uniformity of nipple aspirate fluid (NAF) concentrations within individuals over time supports the contention that hormones in NAF are characteristic of individuals and should therefore be useful biomarkers of continued hormone exposure [23].

Despite the poor correlation between serum and NAF estradiol concentrations, the correlation between serum and NAF progesterone has been reported to be 0.70 and 0.69 in two previous studies [12, 24] suggesting that NAF progesterone may be significantly determined by uptake from serum. If factors within the breast microenvironment can indeed influence the amount of hormone available to breast cancer cells, then it is plausible that local hormone levels will show a stronger relation with cancer risk than circulating hormone levels. Since steroids are capable of diffusion across plasma membranes, and the transfer into or out of cells is not dependent upon transport proteins [25], it is reasonable to assume that the concentration of the steroids in NAF is a reflection of the concentration in the tissue.

The contralateral unaffected breast of women with breast cancer is at increased risk for breast cancer [26, 27], and is a good model of the local breast environment that may influence breast cancer risk. NAF provides a relatively noninvasive sample of the breast environment and allows measurement of an important component of this environment, namely the steroid hormone content.

We obtained NAF samples from the contralateral breasts of breast cancer cases, and from screening mammography controls, using a case-control design with frequency-matching for age, race, and menopause. We confirm previously observed patterns of steroids in NAF during the menstrual cycle and provide new information regarding the relationship of NAF hormones to breast cancer risk.

The authors declare that they have no conflicts of interest.

METHODS

Subjects

Study participants were women with newly diagnosed unilateral breast cancer and screening mammography controls with normal mammograms and breast exams. There were 160 cases and 157 controls in the main study (two premenopausal women did not have menstrual data, and were unavailable for this comparison). For this analysis, only women who provided a NAF samples were included, and women with current or past endocrine disorders or taking exogenous hormones were excluded. The study was conducted at the Lynn Sage Comprehensive Breast Center, under a protocol approved by the Northwestern Institutional Review Board. Consent was obtained from each patient after full explanation of the purpose and nature of all procedures used.

Data and specimens

All subjects completed a health history questionnaire. In premenopausal subjects, the last menstrual period (LMP) and the next menstrual period (NMP) of the cycle in which NAF and blood was obtained were recorded. NAF collection was performed in standard fashion; the breast was warmed, massaged, and a nipple aspirator was used to apply 10 cc of suction followed by manual stripping of the nipple. NAF was collected in calibrated capillary tubes (1 mm = 1uL in volume) until no more could be expressed or more than 10 uL had been collected. The original study design stipulated luteal phase NAF collection, but this quickly proved unfeasible in breast cancer patients who were trying to schedule their treatment procedures. NAF samples were therefore obtained at the subject's convenience, and menstrual cycle phase was carefully assessed in the analyses. Menstrual cycle phase and menopausal status was confirmed post hoc based upon the following criteria: postmenopausal women reported no menstrual period within the last 12 months and had serum follicle stimulating hormone (FSH) levels >30 mIU/ml, estradiol levels <30 pg/ml, and progesterone <3 ng/ml. The menstrual cycle was divided into three phases. Menstrual phase by dates were determined by counting back from the next menstrual period: the follicular phase days -20 through the first day of LMP, mid-cycle days -19 through -12, and luteal phase days -11 through 0. Menstrual phase assignment was confirmed based on serum hormone levels: follicular phase serum estradiol concentrations were <60 pg/ml, and progesterone <3.0 ng/ml; mid-cycle serum estradiol levels >60 pg/ml and progesterone <3.0 ng/ml; luteal phase estradiol levels >30 pg/ml and progesterone >3.0 ng/ml. Only cycles that met these criteria were considered in the analysis of menstrual phase and NAF hormones.

Laboratory analyses

Estradiol was measured in serum by a radioimmunoassay from Beckman Coulter DSL-2700 from Brea, CA. Other analytes, measured by ELISAs from Beckman-Coulter with catalog numbers, were: estrone, DSL-9700; androstenedione, DSL-3800; and DHEA-sulfate, DSL-2700; from Alpcos FSH, 11-FSHU-E01; and DHEA, 20-DHEU-E01; and from Salimetrics testosterone, 1-2402; and progesterone, 1-502-5. Aliquots from a pool of female control serum were inserted into each batch for quality control. The intra-assay and inter-assay %CVs for the serum immunoassays were: estradiol 10.4 and 17.2, FSH 7.2 and 7.6,

progesterone 2.6 and 8.1, estrone 6.6 and 14.0, androstenedione 5.3 and 6.6, testosterone 10.5 and 16.4, DHEA 8.7 and 10.2.

Steroid hormones in NAF were extracted with ethyl acetate-hexane (3:2), containing 250 ng of the internal standard, dexamethasone acetate. The extract was then applied to a 25 x 4.6 mm C₁₈ reversed-phase high-performance liquid chromatography system and eluted with 58% of 15 mmol/L phosphate buffer (pH 6) and 42% of a 50:50 mixture of acetonitrile and methanol. A gradient was started at 40 min to a final concentration of 71% of the second solvent at 50 min. The flow rate was 1.0 ml/min. Each fraction was collected for immunoassays in an automatic fraction collector as previously described in detail [24]. The accuracy, recovery, and precision have been described previously [24]. A preparation of breast cyst fluid (BCF), which is similar in content to NAF, was used as the quality control substance. This was frozen in small aliquots; aliquots were thawed, and one was placed along with a procedural blank between every set of 4 samples. Values were corrected for recovery of the internal standard. Estradiol was measured by a radioimmunoassay from Beckman Coulter and the other steroids were measured by ELISAs, all after HPLC purification.

NAF samples were assayed in 16 batches; each batch contained an equal number of cases and controls, frequency-matched for age, race and menopausal status. Batches 10 through 15 were assayed by a different technician than 1 through 9 and failed largely because of high blank values. They have therefore been excluded from the analysis, leaving 317 subjects whose data are presented in this report. In the 317 evaluated NAF samples, the following numbers of samples yielded measurements in the detectable range for each hormone: 220 (69.4%) for estradiol, 228 (71.9%) for estrone, 230 (72.6%) for testosterone, 225 (71.0%) for androstenedione, 302 (95.3%) for DHEAS, 239 (75.4%) for DHEA, and 287 (90.5%) for progesterone. The intra-assay and inter-assay %CVs of the quality control samples were: estradiol 5.2 and 5.2, estrone 4.4 and 6.7, testosterone 3.0 and 6.6, androstenedione 5.6 and 8.7, DHEA 10.3 and 13.1 and progesterone 11.0 and 13.2.

Statistical analyses

Descriptive characteristics of study participants were summarized using means and standard deviations for continuous variables and counts and frequencies for categorical variables. These variables were compared for pre- and post-menopausal cases and controls using t-tests and Fisher's Exact tests as appropriate.

The concentrations of the steroids were transformed to their natural logarithms to achieve adequate normality for parametric statistical analyses [12, 28]. Steroid concentrations not in the detectable range were omitted from analysis. Data were summarized as medians and interquartile ranges (IQRs) across all batches on their original scale. ANCOVA was used to evaluate hormone concentration differences by menopausal phase adjusted for batch. Partial correlation coefficients were calculated to evaluate correlations of NAF and serum hormone measurements after correction for batch.

Through visual inspection of boxplots and examination of quality control measures there was substantial inter-batch variability; therefore, conditional logistic regression models with

strata defined by batch were used to examine associations between NAF hormones and case-control status. Separate models were analyzed for each hormone. Initially, associations were examined separately for pre- and post-menopausal women with adjustment for menstrual cycle phase (follicular, mid-cycle, luteal) for premenopausal women. Estimated effect sizes had consistent direction and similar magnitude for pre- and post-menopausal women, and so associations were reported using data from all women. In sensitivity analyses, adjustment for a four-category variable combining menstrual stage and phase (premenopausal follicular, premenopausal mid-cycle, premenopausal luteal, postmenopausal) was comparable to a simpler phase adjustment for pre- v. postmenopausal status, and additional adjustment for body mass index (BMI) had little impact on estimated associations. Adjusted results are therefore reported using a simple pre/post-menopausal phase adjustment. Polytomous conditional logistic regression was also used to examine hormone associations of ER positive and ER negative cases versus controls, including adjustment for menopausal status. To confirm results in parametric analyses, a nonparametric analysis was also employed. For each hormone, observed values were ranked within batch in ascending order. Within each batch, the average of the ranks were calculated for cases and controls and then divided by the average number of cases and controls in each batch to measure the proportion of the maximum possible difference in mean ranks for cases and controls observed within batch. An overall test statistic was then calculated as the mean of the batch-specific proportions. Statistical significance of this test statistic was determined by randomly reassigning case-control status within batch, preserving the number of cases and controls within batch, and then recalculating the test statistic. P-values were reported as the proportion of 1000 permutations yielding test statistics as or more extreme than the observed test statistics for each hormone.

RESULTS

Characteristics of the study population

A total of 156 premenopausal and 164 postmenopausal women (2 missing menopausal status) yielding 2 μ L of NAF, assayed in batches 1–9, are included in the present analysis; their characteristics are shown in Table 1. Summary statistics showing they are similar to women with NAF in batches 10–16 are reported in Online Resource-1 Table S1. The mean ages of the women who were included in this analysis were 46 years for those sampled in follicular phase (N=41), 46 years for mid-cycle (N=70), 46 years for luteal phase (N=40), and 57 years for 164 postmenopausal women. The age range of premenopausal subjects in the study was 33 to 61 years, and that of postmenopausal subjects was 42 to 70 years.

For premenopausal women, cases were more frequently parous than controls, $p=0.04$). In postmenopausal women, cases had a significantly higher BMI (31.1 vs 28.0, $p<0.01$) and higher age at menarche (12.7 v. 12.2, $p=0.02$) than controls. The mean volume of NAF did not vary significantly among groups (12.1 ± 12.5 uL in the follicular, 15.1 ± 19.1 uL in the mid-cycle, 20.2 ± 21.9 uL in the luteal phases, and 13.5 ± 15.8 uL in postmenopausal women), with a range of 2.0 to 126. However, in high yielders, collection of >10 μ L depended on the subject's willingness to continue. Sixty percent of subjects produced at least 2 μ L of breast fluid (59% of cases and 62% of controls. The mean cycle sampling day

for the premenopausal women (counting backwards from the date of the next period) was –26 in follicular phase, –14 in mid-cycle, and –9 in luteal phase.

Hormone concentration in NAF and serum

The patterns of hormones in concomitant serum and NAF samples throughout the menstrual cycle were assessed by ANCOVA, adjusted for batch. Confirming earlier work by this group of investigators [24](25) the pattern of estradiol in NAF across the menstrual cycle in this larger study differs significantly from that in serum Table 2. The lower concentrations of NAF estradiol at mid-cycle are not due to differences in volume of NAF or to NAF protein concentrations. While the mean concentration of NAF estradiol was lower after menopause, it was not significantly lower. This result was confirmed in fine needle aspirates of the breast in a recent publication in which NAF estradiol was measured by mass spectrometry after HPLC purification [29]. In general, the concentrations of all steroids except progesterone were more uniform across menstrual and menopausal states in NAF than in serum.

The partial correlations between serum and NAF concentrations of estradiol, estrone, testosterone and androstenedione (Table 3) were all <0.1. The correlation between serum and NAF DHEA was 0.20 ($p = 0.003$). As expected, the correlation of NAF progesterone with serum progesterone was high at 0.49 ($p < 0.001$). Correlations of steroids within NAF are shown in Table 4. Among the potential estradiol precursors, the highest correlation was of NAF DHEA with NAF estradiol ($r = 0.21$, $p = 0.005$). NAF androstenedione was correlated with NAF testosterone but neither of these C₁₉ steroids was correlated with DHEA.

NAF hormones and case-control status

Our primary hypothesis related to the estradiol content of NAF as an indicator of breast cancer risk, but we saw no significant association between NAF estradiol and case-control status (Table 5). However, higher DHEA content in NAF was significantly associated with breast cancer risk ($p = 0.01$). The higher NAF testosterone concentration for cases versus controls also approached significance. Conversely, NAF progesterone concentration was slightly lower in cases compared to controls after adjustment for menopausal status ($p = 0.05$), Table 5. Analyses adjusted for menstrual phase showed similar results, and are not shown. We then analyzed the data using nonparametric methods, and observed similar results in terms of direction of association and statistical significance (Online Resource-1 Table S2). Serum hormone levels after adjustment for menopausal status were not significantly associated with case/control status, except for a marginal negative association for progesterone (Table 6).

We performed polytomous logistic regression to examine potentially unique associations of NAF hormone content for ER positive and ER negative breast cancers. NAF DHEA demonstrated a stronger positive association with ER positive than with ER negative breast cancer (Table 7). NAF progesterone demonstrated an inverse association only with ER negative cancer risk. No other statistically significant associations were found. The serum values are shown in Table 8. Estrogens were not associated with ER positive or negative

cases. Serum DHEA was not associated with ER positive or negative cases versus controls, but serum progesterone, as in NAF, appeared to be negatively associated with ER negative cancer. Adjustment for menstrual phase was not feasible for progesterone since there were 13 pre-menopausal ER negative cases (4 in follicular, 7 in mid-cycle, and 2 in luteal phase).

DISCUSSION

The importance of steroid hormone exposure to breast cancer etiology is clear, but so far has only been studied in terms of systemic exposure, as reflected by reproductive history, circulating steroid concentrations, or exogenous use. The local breast environment has been relatively inaccessible, although a few small studies of breast tissue hormone concentrations have been published [9, 10, 29]. In this study, we focused on NAF as a suitable and available bio-sample of breast tissue hormone levels. Our previous work suggests that the breast estradiol exposure is locally regulated through endogenous synthesis within the breast [12], and that NAF estradiol is more stable over time than serum concentrations [23]. Based on that work, we hypothesized that NAF estradiol content may be a more robust indicator of breast cancer risk than serum estradiol. We performed a case-control study of NAF hormone concentrations in the at-risk but unaffected contralateral breast of incident breast cancer cases compared to screening mammography controls. We reasoned that the unaffected breast of women with unilateral breast cancer is a good high risk model since the risk of future cancer is high (particularly in the absence of systemic therapy) and second cancers in this population have similar hormone receptor characteristics to the index cancer [30]. Additionally, the contralateral breast is not exposed to the local synthesis of hormones by the tumor [31, 32], or the inflammatory consequences of recent diagnostic biopsy. It is possible that tumor itself may have some effect on metabolism, although this appears unlikely [33, 34]. In earlier studies, we have observed that the hormonal environment of both breasts is similar; NAF estradiol, estrone sulfate, DHEA, androstenedione, and progesterone correlations between breasts were 0.61, 0.84 0.65 0.71 and 0.71, respectively (24).

We found that NAF estradiol was not related to breast cancer risk (OR 1.05, 95% CI 0.89–1.23), but there was a positive association between NAF DHEA and risk after adjustment for menopause; OR 1.19, 95% CI 1.04–1.37 for each unit increase in log DHEA concentration. In analyses modeling ER+ and ER– breast cancer, this association remained statistically significant only for ER+ disease (OR 1.18, 95% CI 1.02, 1.36). NAF testosterone was not associated with risk in our study, although data on postmenopausal women from earlier studies suggest an association between serum testosterone and breast cancer risk [15–18]. Our results, in pre and postmenopausal women, are consistent with the serum estradiol findings in premenopausal women from the large EPIC study [19] in which no association was found between pre-diagnostic serum concentrations of estradiol and breast cancer risk, even though a significant interquartile OR of 1.56 (C.I. 1.15–2.13) was found in pre-diagnostic serum testosterone of premenopausal women. The relation of NAF progesterone with breast cancer risk was surprisingly inverse for all cases, with an OR of 0.84 (95% CI 0.71–1.00), and particularly for ER-negative cases (OR 0.69, 95% CI 0.51, 0.92), but the significance of this is limited by the fact that there were only 13 ER-negative premenopausal

cases with measureable NAF progesterone, of whom only 2 were in luteal phase. Among controls, on the other hand, 19 women were sampled in luteal phase.

We found no association of NAF estradiol with the serum estrogens, yet significant correlations were found between NAF estradiol and NAF DHEA. It is interesting that of the C19 potential precursors of estradiol in NAF, DHEA (which is abundant and therefore forms a large precursor pool) was highly significantly related to estradiol, but androstenedione and testosterone were not. The likely independent formation of NAF estrogens from NAF DHEA is suggested by the fact that NAF DHEA was not correlated with either androstenedione or testosterone. While DHEA cannot undergo aromatization [35], it appears that there is compartmentalization of the pathway from DHEA to estrogens in NAF such that the main pools of androstenedione and testosterone are not available for aromatization. This has been described for other steroid biosynthetic systems (37). NAF DHEA was significantly related to serum DHEA, although the fraction accounted for was only 4%. Evidence of 17 β -hydroxysteroid dehydrogenase type 1 activity in the breast is indicated by the fact that androstenedione and DHEA were significant precursors of estradiol. NAF estrone sulfate has also been considered to be an important precursor of estrogens in the breast [36, 37] but was not measured in this study.

Our data, which are the first available data on NAF DHEA levels and risk, show positive associations for DHEA and breast cancer risk after adjustment for menopausal status, confirmed by separate examination of these associations in pre- and postmenopausal women. However, previous studies of serum DHEA have shown mixed results, with a suggestion of differential effects across the menopause (promoting risk in postmenopausal women but offering protection in premenopausal women) [38]. In premenopausal women Helzlsouer et al. [39] found lower mean serum DHEA in cases than in controls, with a relative risk of 0.4. Secreto et al. [40] found similar trends in premenopausal subjects, but postmenopausal cases had higher levels than controls. However, in the Nurses' Health Study [41], women with ER+/PR+ tumors and the highest quartile compared to the lowest quartile of serum DHEA demonstrated a relative risk of 1.6, $P(\text{trend}) = 0.09$, and this was higher in premenopausal than postmenopausal women. Other studies of serum DHEA in postmenopausal women have found positive associations of DHEA and DHEAS with breast cancer risk, with a range of relative risk for DHEAS of 1.69 to 2.8 [15, 17, 18, 42], comparing highest to lowest quartile. We did not observe an association between serum DHEA and risk, despite a significant correlation ($r = 0.45$) between NAF and serum DHEA concentrations.

Most studies have concluded that DHEA is active by either by conversion to 5-androstene-3 β ,17 β -diol, which has a moderate affinity for the estrogen receptor or after its conversion to estradiol [43, 44]. The importance of direct binding of 5-androstene-3 β ,17 β -diol to the estrogen receptor has been demonstrated [45]; and in one study, serum 5-androstene-3 β ,17 β -diol was related to breast cancer risk, with a relative risk of 3.0 [42]. The fact that DHEA had a higher association with cancer risk than estradiol or other androgens is in keeping with Labrie's concept of organ-specific conversion of DHEA to active products [44]; our findings of a relatively high correlation of NAF estradiol with NAF DHEA and other androgens (Table 4) is consistent with conversion of DHEA to estrogens within the

breast. Alternatively, DHEA may act through its own receptors, and specific DHEA receptors have been characterized in liver [46] and T lymphocytes [47].

Surprisingly, we observed inverse associations of both NAF and serum progesterone with breast cancer risk overall, and these associations were stronger in women with ER– disease, and remained significant after adjustment for menopausal status. This must be considered tentative considering the small number of cases in luteal phase, although Haddad [48] found that the SNP rs11571215 of the progesterone receptor was highly associated with ER– breast cancer. Alternatively, a possible explanation for our findings is that the metabolism of progesterone is different in high risk breasts. Wiebe et al. [49] have shown that one metabolite of progesterone, 3 α -hydroxy-4-pregnen-20-one (3 α HP), suppresses proliferation and increases apoptosis in breast cancer cell lines while another metabolite 5 α -dihydroprogesterone (5 α DHP) promotes the growth of mammary cancer. In addition, growth of xenografts of the ER-negative human breast cancer cell line MDA MB 231 was promoted by 5 α DHP and suppressed by 3 α HP in a mouse model [50], and, recently, progesterone-dependent tumorigenesis of transplanted C4HD mouse mammary cells was shown to be inhibited by the 5 α -reductase inhibitor finasteride but tumorigenesis by 5 α DHP was unaffected [51]. This leads to the hypothesis that metabolism of progesterone to 5 α DHP may be favored in the high risk, contralateral breast, decreasing its remaining progesterone content. If this metabolite is retained in normal breast tissue as it is in breast tumor tissue [51], the result would be the promotion of breast cancer despite the lower measured progesterone concentrations. The human breast does have the necessary 5 α -reductase, particularly the SRD5A2 isoform, to produce 5 α DHP [52–54]. The predominant metabolic product of progesterone in the normal breast is 20 α -dihydroprogesterone [55, 56] but 5 α DHP predominates in breast tumors [56]. High affinity, specific, membrane-bound receptors for 5 α DHP have been described in ER positive and negative cell lines [57]. A clinical study also supports a role of 5 α -DHP; breast tumors expressing 5 α -reductase by IHC were more aggressive and the patients had shorter recurrence-free survival time [58]. Further work is justified to investigate the role of progesterone metabolism in the breast.

In summary, in this study there was no association between NAF estradiol and breast cancer risk based on contralateral unaffected breasts of cancer cases versus controls, but we did observe a positive association of NAF DHEA with ER positive cancer. The lack of association of serum DHEA with risk indicates a closer association of NAF than serum DHEA with breast cancer risk in individuals. Although estrogen levels were not significantly associated with cancer risk in our data, the high correlation of estrogens and androgens within the tissue provide evidence for greater availability of estrogen in the unaffected, high risk breast. The negative association of NAF progesterone with ER negative cancer after adjustment for menopausal status must be considered preliminary, and may be explained by the small number of luteal phase ER negative cases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by a grant from the National Institutes of Health, National Cancer Institute R01 CA 120555 to Seema A. Khan, P.I.

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Table 1

Characteristics of 317 women included in final analysis of NAF hormones* .

Characteristic	Premenopausal				Postmenopausal			
	Cases		Controls		Cases		Controls	
	N	Mean (SD) or N (%)	N	Mean (SD) or N (%)	N	Mean (SD) or N (%)	N	Mean (SD) or N (%)
Age	81	45.99 (4.62)	70	45.40 (4.40)	78	56.27 (5.61)	86	56.83 (5.98)
Race	81		70		78		86	
African American		17 (20.99)		16 (22.86)		17 (21.79)		30 (34.88)
Caucasian		58 (71.60)		46 (65.71)		59 (75.64)		51 (59.30)
Other		6 (7.41)		8 (11.43)		2 (2.56)		5 (5.81)
Menstrual Phase	81		70					NA
Follicular		21 (25.93)		20 (28.57)				
Mid-cycle		39 (48.15)		31 (44.29)				
Luteal		21 (25.93)		19 (27.14)				
Menarche Age (yrs)	81	12.68 (1.40)	70	12.58 (1.27)	78	12.65 (1.17)	86	12.18 (1.39)
Parous	81	66 (81.48)	70	46 (65.71)	78	68 (87.18)	86	68 (79.07)
Age of First Pregnancy (yrs)	59	27.47 (6.25)	35	27.69 (6.02)	63	25.76 (7.06)	54	26.22 (6.32)
Pregnancy Number	66	2.74 (1.23)	46	2.59 (1.59)	68	2.69 (1.41)	68	2.62 (1.40)
Ever Lactated	59	47 (79.66)	35	29 (82.86)	63	40 (63.49)	54	37 (68.52)
Duration of Lactation (Months)	59	12.78 (14.79)	35	13.44 (19.29)	63	9.66 (14.56)	54	14.32 (18.53)
Years Since Last Birth	59	14.51 (7.03)	35	13.23 (7.79)	63	25.89 (10.60)	54	27.56 (8.93)
BMI (kg/m ²)	81	27.26 (6.30)	70	27.46 (6.55)	78	31.06 (7.16)	86	27.98 (6.13)
Any Previous Breast Biopsy	81	32 (39.51)	70	6 (8.57)	<0.01	32 (41.03)	86	28 (32.56)
Positive Family History	46	3 (6.52)	25	7 (28.00)	0.03	10 (23.26)	35	13 (37.14)
								0.22

* Two of these 317 women were missing menopausal status. The entire population of NAF yielders numbered 565. Characteristics of the additional 248 NAF yielders are compared to these 317 women in Online Resource Table S1.

Geometric mean concentrations of steroids in NAF and serum by menstrual cycle phase and postmenopause. ANCOVA corrected for batch with Tukey's post hoc testing of comparisons with luteal phase concentrations

Table 2

Steroid	NAF Hormones						
	Menstrual Cycle			4 Post-menopause (N = 155)	P ANCOVA	P 3 v 1	P 3 v 2
	1 Follicular (N = 48)	2 Mid-cycle (N = 68)	3 Luteal (N = 37)				
Estradiol (pg/ml)	251	164	203	159	0.429	NS	NS
Estrone (pg/ml)	481	226	332	263	0.105	NS	NS
Testosterone (ng/ml)	0.28	0.31	0.16	0.23	0.544	NS	NS
Androstenedione (ng/ml)	1.98	1.69	1.97	1.64	0.915	NS	NS
DHEA (ng/ml)	52.6	20.6	61.4	28.4	0.049	NS	NS
Progesterone (ng/ml)	2.23	1.34	12.49	0.119	<0.001	0.089	<0.001
Total protein (mg/ml)	112	115	134	137	0.029	0.335	0.896
Serum Hormones							
Estradiol (pg/ml)	41.8	125	75.6	21.6	<0.001	0.002	<0.001
Estrone (pg/ml)	67.5	119	111	92.2	0.100	NS	NS
Testosterone (ng/ml)	0.23	0.38	0.36	0.34	0.493	NS	NS
Androstenedione (ng/ml)	0.76	0.73	0.72	0.51	0.030	0.965	0.034
DHEA (ng/ml)	5.87	7.00	7.74	5.07	0.002	0.253	0.008
Progesterone (ng/ml)	0.75	1.09	6.90	0.39	<0.001	<0.001	<0.001
FSH (mIU/ml)	12.4	9.08	5.96	75.2	<0.001	0.424	<0.001

Table 3

Partial correlations between concentrations of steroids in serum and NAF after correction for batch

Hormone	N	Correlation	P value
Estradiol	213	0.059	0.348
Estrone	223	0.091	0.185
Testosterone	200	−0.051	0.424
Androstenedione	219	−0.031	0.640
DHEA	232	0.200	0.003
Progesterone	241	0.490	<0.001

Table 4

Partial correlations among NAF products and precursors in NAF after correction for batch

Steroid pair	N	Correlation	P-value
Estradiol & testosterone	159	0.11	0.176
Estradiol & androstenedione	153	0.14	0.078
Estradiol & DHEA	165	0.21	0.005
Estrone & testosterone	164	0.05	0.543
Estrone & androstenedione	167	0.27	<0.001
Estrone & DHEA	182	0.21	0.003
Testosterone & androstenedione	164	0.22	0.001
Testosterone & DHEA	165	0.07	0.314
Androstenedione & DHEA	170	0.10	0.175

Table 5

NAF steroid concentrations in cases and controls and breast cancer risk estimates

Steroid	Cases Median (IQR ^{**})	Controls Median (IQR ^{**})	Univariate Analysis			Multivariate (adjusted for menopause)		
			N	OR* (95% CI)	P	N	OR* (95% CI)	P
Estradiol (pg/ml)	162.8 (67.5,461.4)	188.0 (75.2,533.4)	220	1.05 (0.90, 1.24)	0.52	218	1.05 (0.89, 1.23)	0.56
Estrone (pg/ml)	333.6 (132.8,759.4)	255.8 (94.2,763.1)	228	1.16 (0.98, 1.38)	0.09	226	1.16 (0.97, 1.37)	0.10
Testosterone (ng/ml)	0.26 (0.11,0.78)	0.19 (0.085,0.62)	230	1.17 (0.98, 1.40)	0.09	228	1.19 (0.99, 1.42)	0.07
Androstenedione (ng/ml)	1.70 (0.66,6.22)	1.58 (0.82,7.22)	225	0.97 (0.83, 1.13)	0.69	223	0.98 (0.84, 1.14)	0.77
DHEA (ng/ml)	32.6 (11.3,167.2)	20.9 (5.4,70.6)	239	1.19 (1.04, 1.36)	0.01	237	1.19 (1.04, 1.37)	0.01
DHEAS (µg/ml)	26.5 (78.7,66.5)	17.3 (35.8,42.0)	302	1.09 (0.98, 1.20)	0.12	300	1.09 (0.98, 1.20)	0.12
Progesterone (ng/ml)	3.99 (1.68,9.80)	5.51 (1.80,18.79)	287	0.89 (0.76, 1.04)	0.14	285	0.84 (0.71,1.00)	0.05

* OR is calculated per unit increase in the log-transformed hormone concentration.

** IQR is between the 25th and 75th percentiles

Table 6

Serum steroid concentrations in cases and controls and breast cancer risk estimates

Steroid	Cases Median (IQR ^{**})	Controls Median (IQR ^{**})	Univariate Analysis			Multivariate (Adjusted for menopause)		
			N	OR [*] (95% CI)	P	N	OR [*] (95% CI)	P
Estradiol (pg/ml)	32.8 (21.5,73.9)	29.5 (16.1,64.4)	310	1.26 (0.98, 1.62)	0.07	310	1.28 (0.89, 1.86)	0.18
Estrone (pg/ml)	67.6 (48.9,104.2)	67.3 (45.8,95.0)	309	1.33 (0.90, 1.97)	0.16	307	1.31 (0.88, 1.94)	0.19
Testosterone (ng/ml)	0.32 (0.22,0.48)	0.30 (0.20,0.40)	256	1.20 (0.87, 1.66)	0.27	254	1.20 (0.86, 1.66)	0.28
Androstenedione (ng/ml)	0.49 (0.34,0.81)	0.50 (0.30,0.83)	310	1.08 (0.75, 1.56)	0.68	310	1.01 (0.69, 1.49)	0.96
DHEA (ng/ml)	5.02 (3.51,7.22)	4.73 (3.39,7.57)	310	0.95 (0.66, 1.35)	0.76	310	0.90 (0.62, 1.29)	0.56
Progesterone (ng/ml)	0.33 (0.16,1.36)	0.44 (0.21,1.70)	296	0.92 (0.79, 1.07)	0.29	296	0.84 (0.70, 1.00)	0.05

* OR is calculated per unit increase in the log-transformed hormone concentration.

** IQR is between the 25th and 75th percentiles.

Associations of NAF steroid concentrations with ER positive and ER negative breast cancer (adjusted for menopausal status).

Table 7

Analyte in NAF	Control N	ER Positive			ER Negative		
		N	OR (95% CI)	P	N	OR (95% CI)	P
Estradiol (pg/ml)	109	86	1.06 (0.91, 1.23)	0.44	22	0.88 (0.67, 1.17)	0.39
Estrone (pg/ml)	110	87	1.13 (0.95, 1.34)	0.17	28	1.15 (0.89, 1.49)	0.27
Testosterone (ng/ml)	113	87	1.10 (0.94, 1.29)	0.23	27	1.15 (0.91, 1.47)	0.24
Androstenedione (ng/ml)	106	87	1.04 (0.89, 1.21)	0.63	29	0.83 (0.65, 1.06)	0.13
DHEA (ng/ml)	119	92	1.18 (1.02, 1.36)	0.02	25	1.13 (0.90, 1.42)	0.29
DHEAS (µg/ml)	143	121	1.07 (0.96, 1.19)	0.21	35	1.16 (0.96, 1.40)	0.13
Progesterone (ng/ml)	138	113	0.89 (0.74, 1.06)	0.19	33	0.69 (0.51, 0.92)	0.01

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Table 8
Association of serum steroids with ER positive and ER negative breast cancer (adjusted for menopausal status).

Analyte in Serum	Control N	ER Positive			ER Negative		
		N	OR (95% CI)	P	N	OR (95% CI)	P
Estradiol (pg/ml)	155	120	1.20 (0.84, 1.72)	0.32	34	1.24 (0.70, 2.19)	0.46
Estrone (pg/ml)	154	120	1.29 (0.87, 1.90)	0.21	32	1.32 (0.72, 2.42)	0.36
Testosterone (ng/ml)	130	97	1.21 (0.86, 1.71)	0.27	27	1.25 (0.73, 2.15)	0.41
Androstenedione (ng/ml)	155	120	0.93 (0.62, 1.39)	0.73	34	1.24 (0.67, 2.30)	0.49
DHEA (ng/ml)	155	120	0.99 (0.68, 1.42)	0.94	34	0.67 (0.37, 1.20)	0.17
Progesterone (ng/ml)	147	114	0.87 (0.72, 1.05)	0.15	34	0.71 (0.52, 0.97)	0.03