

## Research Article

# Patterns of Sex Steroid Hormones in Nipple Aspirate Fluid during the Menstrual Cycle and after Menopause in Relation to Serum Concentrations

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## Abstract

Previous studies have shown that progesterone concentrations in serum and nipple aspirate fluid (NAF) are significantly correlated in premenopausal women, but estradiol concentrations are not. We therefore sought to ascertain the patterns of both steroids in NAF throughout the menstrual cycle and in postmenopausal women. Simultaneous samples of blood and NAF were obtained from 40 premenopausal and 16 postmenopausal women. Premenopausal samples were backdated from the following menstrual period. Steroids were purified by high-performance liquid chromatography before quantification by immunoassays. Serum steroids and NAF progesterone followed the expected pattern across the menstrual cycle, with a midcycle peak of estradiol and a midluteal peak of progesterone. However, the estradiol peak in NAF occurred about a week after the serum peak in the midluteal phase, when serum estradiol had declined to less than half the value at midcycle. NAF estrone was also elevated at the midluteal phase. Potential estrogen precursors androstenedione, estrone sulfate, and dehydroepiandrosterone sulfate declined in NAF from midcycle to the midluteal phase as NAF estradiol was increasing. Progesterone concentrations were significantly lower in NAF in postmenopausal women than in premenopausal women, but estrogen concentrations were not. This is the first description of the temporal relationships of sex steroids in NAF and serum relative to the menstrual cycle. These results provide insights into the lack of correlation of NAF and breast tissue estrogens with serum estrogens, and generate new hypotheses. *Cancer Epidemiol Biomarkers Prev*; 19(1); 275–9. ©2010 AACR.

## Background

Nipple aspirate fluid (NAF) is a potentially useful biosample that promises to provide a window into the local endocrine and protein environment of the breast. However, the relation between serum and breast concentrations of important hormones is not straightforward, and needs to be better understood for interpretation of data on NAF constituents. Although the serum and NAF concentrations of progesterone seem to follow a similar pattern (1, 2), a number of studies have failed to show a relationship between estrogen concentrations in serum, and those in NAF (1, 2) or breast tissue (3–5). The reason for this is not clear. One possibility is that, even in premenopausal women, local biosynthesis of estrogens in the breast contributes significantly to the levels in the breast. There is good

evidence of local biosynthesis of estrogens in breast tissue of postmenopausal women: a number of studies have shown that the steroid precursors required for estradiol synthesis are present in breast tissue (6, 7), and high estrogen concentrations exist in NAF (8) and breast tissue (4–6, 9) of postmenopausal women in the presence of dramatically lower concentrations of estrogens in plasma.

We have previously reported measurements of NAF and plasma/serum hormone concentrations in midfollicular and midluteal phase samples from healthy premenopausal women, and have shown that the fluctuation of hormone content is smaller in NAF than in serum (1), but this has not been extended to the entire menstrual cycle. Certainly, the magnitude and pattern of this fluctuation remains important if NAF hormones are to be used as biomarkers of breast cancer risk and prevention. The determinants of high breast estrogen levels also have implications for breast cancer causation and may point to strategies of breast cancer prevention. In an effort to better understand the patterns of the major sex steroids (estradiol and progesterone) concentrations in NAF vis-a-vis serum relative to menstrual and menopausal status, we have now analyzed NAF and serum samples collected from healthy postmenopausal women, and from premenopausal women throughout the menstrual cycle.

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## Materials and Methods

### Subjects

Healthy women presenting for routine breast surveillance at the Lynn Sage Comprehensive Breast Center of Northwestern University were recruited under an International Review Board approved protocol. There were two groups of subjects, all of whom had simultaneously donated NAF and blood. There were 40 premenopausal women with documented information on the day of the menstrual cycle when samples were collected; this included the date of the last menstrual period, and either the date of the next menstrual period or the average length of the cycle. Their average age was 40 y, the mean body mass index was 27.6, and 76% were of European extraction. The average menstrual cycle length of these women was 28 d. These premenopausal women were grouped according to the following criteria: follicular phase of the menstrual cycle ( $N = 7$ ),  $-28$  through  $-19$  d from the next menstrual period; midcycle ( $N = 9$ ),  $-18$  through  $-13$  d from menses; luteal phase ( $N = 15$ ),  $-12$  through  $-6$  d from menses; and late luteal ( $N = 9$ ),  $-5$  through  $0$  d from menses. There were 18 postmenopausal women with an average age of 52 y and a mean body mass index of 26.8, and 55% were of European origin.

### NAF Collection

Moist heat was applied to the breasts, and 2.5% lidocaine/2.5% prilocaine cream (Emla, AstraZeneca, Wilmington, DE) was applied to the nipple and areola area for anesthesia. Fluid was obtained by applying a partial vacuum with a syringe-like device (Cytoc Corp., Boxborough, MA). The fluid was collected from  $\geq 1$  ducts in a calibrated capillary tube (1.0 mm in length = 1.0  $\mu$ L volume). The sample was placed on ice and brought to the laboratory, where the sample was measured and flushed out with 200  $\mu$ L of PBS into a small Eppendorf tube, and then frozen at  $-80^{\circ}\text{C}$ .

### Analysis of Compounds in the Aqueous Fraction

Cathepsin D, DHEA sulfate, and estrone sulfate were measured directly by commercial immunoassays in the residual aqueous fraction of each sample. Previous work has shown that the extraction of the steroid hormones by ethyl acetate:hexane (3:2) does not interfere with the immunoassay of the hormones in the aqueous fraction (10).

### Analysis of Steroids in NAF

Steroids were extracted into water-saturated ethyl acetate:hexane (3:2) containing 250 ng of the internal standard dexamethasone acetate. The extract was then applied to a 25 cm  $\times$  4.6 mm C18 reversed-phase high-performance liquid chromatography column, and eluted with 58% of 15 mmol/L phosphate buffer (pH 6) and 42% of a 50:50 mixture of acetonitrile:methanol. A gradient was started at 40 min to a final concentration of 71%

of the acetonitrile:methanol solvent at 50 min. The flow rate was 1.0 mL/min. The retention times of estradiol, estrone, testosterone, androstenedione, and progesterone were 33, 38, 46, 48, and 57 min, respectively. A single fraction containing estrone and androstenedione was collected, and the fraction was divided equally for the assay of these steroids. Each 4-mL fraction was collected in an automated fraction collector, evaporated at  $50^{\circ}\text{C}$  in a water bath under nitrogen, and assayed by specific immunoassays. Estradiol, estrone, testosterone, androstenedione, and progesterone were assayed by radioimmunoassays from Diagnostic Systems Laboratories. Serum hormones were also assayed with the appropriate assay kits from Diagnostic Systems Laboratories. All methods were validated for accuracy, recovery, and precision.

### Accuracy

An estradiol assay with high-performance liquid chromatography purification was compared with a solvent partition method for isolation of phenolic steroids. We have used the latter method for a number of studies beginning in 2004 (10). Samples were prepared in buffer that was used to dilute the NAF. The calculated concentrations from high-performance liquid chromatography and the partition method were compared with the expected concentrations over a 32-fold dilution. For each steroid the response was linear and closely approximated the expected concentrations for both methods. The correlation coefficients between the reference preparations and the measured steroids over this dilution range were all  $>0.997$ .

### Recovery

Known amounts of each of the steroids were added to samples of a pool of breast cyst fluid (BCF) that was used as the quality control preparation for the study. The concentration of each of the steroids in the BCF was measured, and the sum of the endogenous and added steroids was calculated. This was compared with the mean value of the assayed total concentration of the spiked pool. Table 1 shows the comparison.

### Precision

All samples were assayed for each analyte in a single assay. The intra-assay percent coefficient of variation (CVs) in NAF were: for estradiol, 13.3%; for estrone, 13.0%; for testosterone, 7.5%; for androstenedione, 11.2%; for progesterone, 8.2%; for estrone sulfate, 9.6%; for DHEA sulfate, 9.1%; and for cathepsin D, 7.2%. In serum the percent CVs were all  $<8\%$ .

### Statistical Methods

The concentrations of the steroids were transformed to their natural logarithm values. This procedure gives adequate normalization of such data for parametric statistical analyses (10). Data are displayed as the geometric means for each group. Differences between the groups representing the four phases of the menstrual cycle were

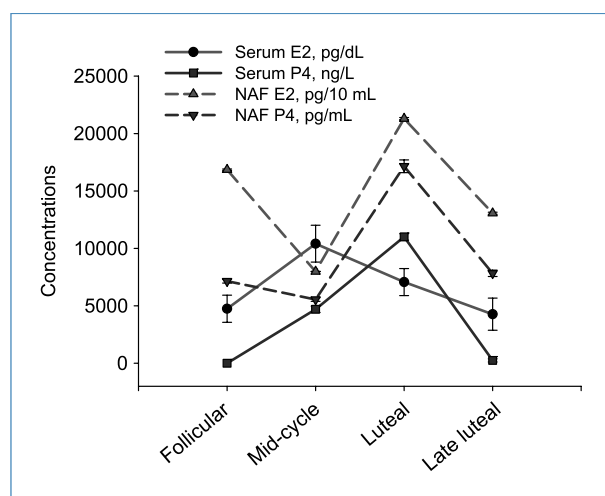
**Table 1.** Recovery of steroids from breast cyst fluid after purification by HPLC

	Estradiol	Testosterone	Estrone	Androstenedione	Progesterone
BCF (pg/mL)	344	4,263	2,525	30,673	8,424
Amount of steroid added (pg)	276	1,696	1,987	14,801	7,768
Sum BCF + standard (pg/mL)	620	5,959	4,512	45,474	16,192
Assayed spiked BCF (pg/mL)	695	6,376	4,243	42,930	14,944
Difference from spiked	10.8%	6.5%	6.3%	-5.9%	-8.4%

assessed by paired *t* tests. Differences between premenopausal and postmenopausal women were tested by group *t* tests.  $P \leq 0.05$  was accepted as significant.

## Results

The serum concentrations of estradiol and progesterone in the group of premenopausal women are shown in Fig. 1. Estradiol reached peak concentrations at midcycle, and progesterone reached a peak in the luteal phase as expected. A box plot of the data is shown in Fig. 2. Concentrations of the sex steroids, steroid precursors, and cathepsin D obtained simultaneously in NAF of the same patients are shown in Table 2. In NAF samples, progesterone reached a mean peak concentration in the luteal phase that was significantly higher than at midcycle (Table 2), as expected. The peak concentrations of estradiol and estrone were shifted from midcycle to the luteal phase; estradiol was significantly higher at the luteal phase than at midcycle (Table 2). Estradiol concentrations in NAF were significantly lower at midcycle than in the luteal phase of the menstrual cycle (Table 2). Serum estradiol was highest at midcycle, with a serum:NAF ratio of 1:6.7, whereas in the midluteal phase this



**Figure 1.** Serum and NAF concentrations of estradiol and progesterone from subjects retrospectively grouped by phase of the menstrual cycle. Units are chosen to fit within the figure to show the relationships. Vertical bars, SE.

ratio was 1:31.4. In contrast, androstenedione and estrone sulfate, which may be considered precursor steroids, tended to decline from midcycle to the luteal phase in NAF (Table 2).

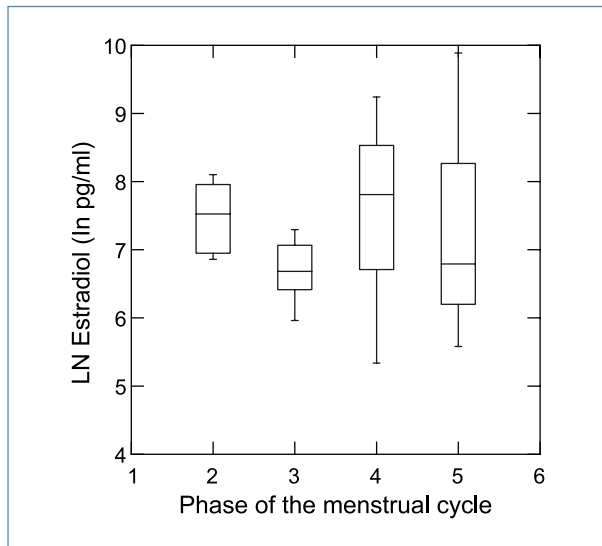
There was no difference between the estradiol levels in NAF of the follicular versus the luteal phase of the menstrual cycle, nor between estradiol in the luteal phase and NAF of postmenopausal women (Table 2). NAF progesterone was greater in the luteal phase than at midcycle and the follicular phase, or the NAF of postmenopausal women. NAF progesterone in postmenopausal women was detectable and was similar to that in the follicular phase of the premenopausal women (Table 2).

In premenopausal women, the Pearson correlations between serum and NAF concentrations at the midluteal phase were as follows: estradiol, 0.173 ( $P = 0.389$ ); estrone,  $-0.230$  ( $P = 0.248$ ); testosterone, 0.122 ( $P = 0.544$ ); and progesterone, 0.688 ( $P = 0.000$ ). Thus, only progesterone concentrations showed a strong and highly significant correlation between serum and NAF. Whereas serum progesterone and NAF estradiol both increased from midcycle to the midluteal phase, the correlation between individual values was not significant.

In postmenopausal women, the correlations between the serum and NAF hormone concentrations were as follows: estradiol, 0.144 ( $P = 0.473$ ); estrone,  $-0.230$  ( $P = 0.248$ ); testosterone, 0.235 ( $P = 0.238$ ); and progesterone, 0.618 ( $P = 0.001$ ). Here also, only progesterone was correlated with NAF and serum.

## Discussion

This is the first report of sex steroid variation in NAF throughout the menstrual cycle, with comparisons with concentrations in NAF of postmenopausal women, an issue of considerable relevance to the use of these measurements as biomarkers of risk or of the success of prevention strategies. We had two objectives: to define the pattern of NAF hormones relative to serum in menstruating women in greater detail, and to gain a better understanding of the cyclical pattern of NAF hormones in premenopausal women and their relative variability compared with postmenopausal women. Our results show several novel insights into these patterns of variation and confirm some previous findings. We find significant differences in NAF estradiol and progesterone



**Figure 2.** Box plot of natural log of estradiol values in NAF. Phases are as follows: 2, midfollicular phase; 3, midcycle; 4, midluteal phase; and 5, late luteal phase of the menstrual cycle. The lowest value is at midcycle.

patterns across the menstrual cycle, confirming the expectation that future studies in premenopausal women will need to specify NAF collection in a specific menstrual cycle phase or collect detailed menstrual cycle information to adjust for the phase of NAF sampling. Our data show clearly that there is a delay in peak estradiol concentrations in NAF in the luteal phase, as opposed to serum, the peak of which occurs at midcycle.

This delay in the peak concentrations of the estrogens explains the previously observed lack of correlation between serum and NAF estradiol (1, 2), which is seen again in the present study, and has been observed between serum and tissue concentrations of estradiol by others (3, 5, 11). However, the association between serum progesterone and NAF hormones has not been examined previously in detail. We find that both NAF

progesterone and NAF estradiol follow serum progesterone concentrations. The reason for this observation will require further study.

The uptake of steroid hormones into tissues has been shown many years ago to be by diffusion; it is not a facilitated process nor is a transporter system involved. The concentration of  $^3\text{H}$ -estradiol in the uterus and other tissues was shown to be dependent on retention by high-affinity binding proteins in the tissues that retained the hormone (12). This work has been confirmed by many investigators. The cause of the decrease in estrogen content in NAF at midcycle is unknown. Estrogen receptor  $\alpha$  content in the breast is relatively low, and estrogen receptor content declines through the cycle (13), with lower expression seen in the luteal than in the follicular phase. Therefore, despite the high preovulatory concentrations of estradiol in serum, it is unlikely that this results in a decrease in estrogen receptor levels sufficient to account for the lack of uptake in the breast observed. Estrogen receptor type II binding sites are more abundant, but the concentration of this binding protein is not decreased by an increase in estradiol (14). The only other obvious change at this time of the cycle is the known surge of gonadotropins at midcycle. There is evidence for gonadotropin receptors in the breast, at least in breast cancer cells (15), and human chorionic gonadotropin has been shown to decrease proliferation and invasion of MCF-7 cells in xenograft models (15), and to induce apoptosis in breast cancer cell lines (16). The mechanism by which activation of the luteinizing hormone/human chorionic gonadotropin receptor brings about these effects is not known, nor is it known whether suppression of estradiol is a component of this process. Future studies should examine this possibility, which could have important implications for the effects of gonadotropins on estrogen retention in postmenopausal women.

The estrogen content of NAF increased rapidly after the midcycle suppression. The data on androstenedione, estrone sulfate, and DHEA sulfate were consistent with the increased conversion of these potential substrates to

**Table 2.** Differences in steroid and cathepsin D levels in NAF between phases of the menstrual cycle, and between premenopausal and postmenopausal women

Steroid	NAF hormone concentrations				P-value for differences		
	Follicular	Midcycle	Luteal	Postmenopausal	Follicular vs luteal	Midcycle vs luteal	Postmenopausal vs luteal
Androstenedione (pg/mL)	7,406	7,801	5,024	7,692	0.394	0.646	0.172
Cathepsin D (ng/mL)	5,590	1,327	2,186	1,912	0.362	0.250	0.712
DHEA sulfate ( $\mu\text{g/dL}$ )	3,182	5,262	1,660	301	0.698	0.174	0.109
Estradiol (pg/mL)	1,685	799	2,130	1,213	0.662	<b>0.014</b>	0.133
Estrone (pg/mL)	3,797	1,929	3,519	6,674	0.802	0.216	0.178
Estrone sulfate (ng/dL)	3,900	2,060	1,470	1,361	0.328	0.419	0.677
Progesterone (pg/mL)	7,143	5,552	17,150	6,995	<b>0.034</b>	<b>0.013</b>	<b>0.005</b>
Testosterone (pg/mL)	1,307	2,044	2,069	2,336	0.352	0.985	0.845

estrogens in the luteal phase of the cycle. A change in the rate of conversion of estrogen precursors could account for the significant increase in estrogen concentrations in the midluteal phase of the menstrual cycle. However, it would seem that a stimulus should be required for this increased activity. Alternatively, vascular permeability factor/vascular endothelial growth factor has been shown to increase in the extracellular fluid of the normal breast by 2-fold from the follicular to the luteal phases of the menstrual cycle, and the higher concentration of vascular permeability factor in the luteal phase may increase the rate of uptake of estrogens from serum (17). Vascular permeability factor is known to be stimulated by progesterone and estradiol in the uterus (18), so if a similar process is active in the breast, this could act as a feed-forward system. Estradiol, but not progesterone, has been correlated with vascular endothelial growth factor levels in the extracellular fluid of the breast in premenopausal women (17). Other luteal phase components, such as inhibin, should be considered in further investigations of this phenomenon. Finally, it is possible that the increased availability of progesterone in the luteal phase may directly induce expression or activity of the enzymatic machinery responsible for estradiol synthesis in the breast, but this too is speculative because the influence of progesterone on this process has received little (if any) attention.

## References

- Chatterton RT, Jr., Geiger AS, Gann PH, Khan SA. Formation of estrone and estradiol from estrone sulfate by normal breast parenchymal tissue. *J Steroid Biochem Mol Biol* 2003;86:159–66.
- Gann PH, Geiger AS, Helenowski IB, Vonesh EF, Chatterton RT. Estrogen and progesterone levels in nipple aspirate fluid of healthy premenopausal women: relationship to steroid precursors and response proteins. *Cancer Epidemiol Biomarkers Prev* 2006;15:39–44.
- Thijssen JH, Blankenstein MA, Miller WR, Milewicz A. Estrogens in tissues: uptake from the peripheral circulation or local production. *Steroids* 1987;50:297–306.
- Thijssen JHH, Blankenstein MA. Uptake and metabolism of estradiol by normal and abnormal breast tissues. *Ann N Y Acad Sci* 1990;586:252–8.
- Geisler J. Breast cancer tissue estrogens and their manipulation with aromatase inhibitors and inactivators. *J Steroid Biochem Mol Biol* 2003;86:245–53.
- Blankenstein MA, Maitimu-Smelee I, Donker GH, et al. Tissue androgens and the endocrine autonomy of breast cancer. *J Steroid Biochem Mol Biol* 1992;43:167–71.
- Thijssen JH, Blankenstein MA. Endogenous oestrogens and androgens in normal and malignant endometrial and mammary tissues. *Eur J Cancer Clin Oncol* 1989;25:1953–9.
- Chatterton RT, Jr., Geiger AS, Mateo ET, Helenowski IB, Gann PH. Comparison of hormone levels in nipple aspirate fluid of pre- and postmenopausal women: effect of oral contraceptives and hormone replacement. *J Clin Endocrinol Metab* 2005;90:1686–91.
- Thijssen JH, Blankenstein MA, Daroszewski J, Milewicz A. Steroids in normal and neoplastic breast tissues. *Ann N Y Acad Sci* 1990;595:222–6.
- Chatterton RT, Jr., Geiger AS, Khan SA, et al. Variation in estradiol, estrogen precursors, and estrogen-related products in nipple aspirate fluid from normal premenopausal women. *Cancer Epidemiol Biomarkers Prev* 2004;13:928–35.
- Thijssen JH, van Landeghem AA, Poortman J. Uptake and concentration of steroid hormones in mammary tissues. *Ann N Y Acad Sci* 1986;464:106–16.
- Jensen EV, Jacobsen HI. Basic guides to the mechanism of estrogen action. *Recent Prog Horm Res* 1962;18:387–414.
- Battersby S, Robertson BJ, Anderson TJ, King RJB, McPherson K. Influence of menstrual cycle, parity and oral contraceptive use on steroid hormone receptors in normal breast. *Br J Cancer* 1992;4:601–7.
- Clark JH, Watson CS, Markaverich BM, Syne JS, Panko WB. Heterogeneity of estrogen binding sites in mammary tumors. *Breast Cancer Res Treat* 1983;3:61–5.
- Janssens JP, Russo J, Russo I, et al. Human chorionic gonadotropin (hCG) and prevention of breast cancer. *Mol Cell Endocrinol* 2007;269:93–8.
- Lopez D, Sekharam M, Coppola D, Carter WB. Purified human chorionic gonadotropin induces apoptosis in breast cancer. *Mol Cancer Ther* 2008;7:2837–44.
- Dabrosin C. Positive correlation between estradiol and vascular endothelial growth factor but not fibroblast growth factor-2 in normal human breast tissue *in vivo*. *Clin Cancer Res* 2005;11:8036–41.
- Ancelin M, Buteau-Lozano H, Meduri G, et al. A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF189 regulates angiogenesis and vascular permeability in human uterus. *Proc Natl Acad Sci U S A* 2002;99:6023–8.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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