Cellular and hormonal content of breast nipple aspirate fluid in relation to the risk of breast cancer

Seema A Khan^{1†} &
Robert T Chatterton Jr²

[†]Author for correspondence

¹Department of Surgery &
the Robert H Lurie

Comprehensive Cancer of
Northwestern University,
301 East Superior Street,
Chicago, IL, USA

Tel.: +1 312 503 2112;

Fax: +1 312 503 2555;

E-mail: SKhan@nmh.org

²Departments of Obstetrics &
Gynecology & Pathology,
Feinberg School of Medicine
& the Robert H Lurie
Comprehensive Cancer
Center of Northwestern
University, 710 N Fairbanks
Court, Chicago, IL, USA

Tel.: +1 312 503 5272;

Fax: +1 312 503 0389;

E-mail: chat@

northwestern.edu

In research settings, the measurement of serum and urine hormone concentrations has shown modest positive relationships with breast cancer risk in postmenopausal women. However, the local production of sex steroids in the breast is a significant contributor to the hormonal environment of the breast. Nipple aspiration fluid provides a window into this environment and allows the measurement of hormone and protein content which may show stronger relations to breast cancer risk, and therefore enable both more accurate risk assessment, and the use of preventive measures directed to the lowering of local breast hormonal exposure

Breast cancer risk can be attributed to three broad categories of risk factors: increasing age, lifetime estrogen exposure (endogenous and exogenous) and genetic susceptibility. These major factors play a critical role in breast epithelial transformation and converge in the phenotypic expression of proliferative precursor lesions of the breast epithelium. These are observed in the unaffected breasts of women who are genetically susceptible (e.g., *BRCA* mutation carriers), as well as women at risk for the so-called 'sporadic' breast cancer. This article will focus on aspects of estrogen exposure that may be important in determining the local endocrine environment of the breast, with a discussion of other determinants of breast cancer risk to the extent that they intersect with the hormonal environment.

Estrogen-related breast cancer risk factors

A wide array of established risk factors for breast cancer relates to endogenous lifetime estrogen exposure. These include young age at menarche, late age at first full-term pregnancy, no exposure to lactation and late age at menopause. Other well-established risk factors also appear to operate through the endocrine axis: postmenopausal obesity is associated with increased aromatization of androgenic precursors in adipose tissue, lower sex hormone-binding globulin and higher free estradiol [1]. Physical activity in adolescence delays menarche and the onset of ovulatory cycles; later in life, it protects against obesity and may operate through other mechanisms as well [2]. Moderate to heavy alcohol consumption is associated with higher circulating sex steroid levels and may retard the hepatic metabolism of hormones [3].

In general, the contribution of these individual risk factors to overall risk is modest, and relative risk estimates for each of these factors from most studies are in the range of 1.5 to 2.0. It is therefore difficult to apply this information to individual risk estimation unless it is incorporated into multifactorial statistical models, the prototype of which is the Gail model [4], validated in the Breast Cancer Prevention Trial [5]. Although use of the Gail model has led to very precise prediction of rates of breast cancer occurrence in groups of women (i.e., it is well-calibrated for populations) [5], the ability to identify individual women who will develop breast cancer (in other words its discriminatory ability) remains poor [6]. Other risk-assessment models have been introduced [7], and are in the process of being validated, but information of this type does not provide good discriminatory ability, and the resulting gap has led to intense interest in biomarkers that may perform better at the individual level. Systemic levels of sex steroids are the most obvious group of biomarkers, and have been extensively studied.

Systemic sex steroid levels & breast cancer risk

The pathways of sex steroid biosynthesis are shown in Figure 1. Studies of circulating sex steroid levels and breast cancer risk have necessarily focused on postmenopausal women, since the menstrual cycle variations of premenopausal women have been a significant barrier to such investigations. Among postmenopausal women, correlations between serum estrogen levels and breast cancer risk are evident in almost all of the larger studies; with relative risk estimates ranging from 1 to 4 for several of the estrogenic

Keywords: breast cancer, cytologic atypia, estradiol, estrogen-response proteins, hormones, nipple aspiration fluid, risk

future
medicine part of
fsg

Figure 1. The sex steroid biosynthesis pathway.

The enzymes that are present in the normal breast include aromatase. Thus, androgen precursors from serum that originate from the ovary or adrenal, or those that may be synthesized in the breast, can be converted to estrone and estradiol. Androstenedione is probably the more important androgen precursor, but testosterone may also contribute. The conversion of estrone to estradiol is essential for a biological effect. Thus, 17 β -HSD-1 is essential and may be the rate-limiting step. The back reaction from estradiol to estrone is catalyzed by the type 2 enzyme. Under most conditions this enzyme predominates over type 1. Sulfatase activity is high in breast tissues and may be important in the conversion of estrone sulfate to estrone. Conversion of DHEA sulfate to DHEA may also serve as a source of estrogen but, under most circumstances, the activity of 3 β -HSD is very low in breast tissue. Progesterone is not known to be synthesized in the breast. DHEA: Dehydroepiandrosterone; HSD: Hydroxysteroid dehydrogenase.

hormones studied [8–10]. In particular, the results of a nested case–control analysis of the Nurses Health Cohort showed the strongest effects for ES, dehydroepiandrosterone sulfate (DHEAS) and estradiol (relative risk [RR]: 4.34, 4.15 and 3.53, respectively), with a surprisingly weak effect of free estradiol (RR: 1.47), which previously had been postulated to be the most bio-available to the breast [11]. An overview analysis of studies published up to 1997 concluded that serum estradiol levels in postmenopausal women who develop breast cancer are approximately 15% higher than in those who do not [12]. Among users of postmenopausal hormones, modest associations between free estradiol, free testosterone, and sex hormone-binding globulin and breast cancer risk were observed in a nested case–control study within the Nurses' Health Study (NHS) cohort [13].

In premenopausal women, the best attempt so far was a recent case–control study nested within the European Prospective Investigation into Cancer and nutrition (EPIC) cohort, which evaluated serum hormone data from 370 premenopausal cases and 726 matched controls [14]. Blood was collected an average of 3 years prior to diagnosis from cases. The menstrual cycle timing at blood

collection was assessed through the use of menstrual dates, either prior to or following the time of blood draw. In the EPIC study, no increase in risk was seen with increasing quartiles of serum estradiol, or serum estrone, although these investigators did find an increased breast cancer risk in women with the highest quartile of serum testosterone compared with the lowest (odds ratio [OR]: 1.7; 95% confidence interval [CI]: 1.2–2.6). Elevated risks were also found for other androgens: androstenedione (OR: 1.56; 95% CI: 1.1–2.3) and DHEAS (OR 1.48; 95% CI: 1.0–2.1). However, prior studies on adrenal androgens and breast cancer risk in premenopausal women have shown conflicting results, and in particular, an earlier case–control study nested within the Nurse' Health Study showed no increase in breast cancer risk for plasma (dehydroepiandrosterones) DHEA and DHEAS [15]. A more recent nested case–control analysis from the NHS II showed that women in the highest quartiles of follicular (but not luteal) total and free estradiol levels had significantly increased risks of breast cancer (RR: 2.1 [95% CI: 1.1–4.1] and RR 2.4 [95% CI: 1.3–4.5], respectively) [16]. The findings were more marked for invasive tumors and for estrogen receptor and progesterone receptor-positive (ER⁺/PR⁺) tumors.

The highest quartiles of total and free testosterone and androstenedione in both menstrual cycle phases were associated with significant increases in risks of invasive and ER⁺/PR⁺ cancers (e.g., RR of invasive cancers for luteal total testosterone levels: 2.0 [95% CI: 1.1–3.6] and RR of ER⁺/PR⁺ cancers: 2.9 [95% CI: 1.4–6.0]). The greater risk associated with androgens may be more apparent because androgens are more constant during the menstrual cycle than estrogens.

The negative findings relative to serum estradiol in the EPIC cohort, and other studies of premenopausal women need to be interpreted in view of data that a single measure of serum estradiol does not adequately capture the average estradiol exposure of premenopausal women. A study of serum hormone reproducibility in premenopausal women found that the intraclass correlation coefficient of serum estradiol was 0.06 [17]. The measurements were performed at two time points 1 year apart, on the same day of the menstrual cycle, at the same time of day, after a 12-h fast. Similarly, there was no correlation in another study between salivary estradiol levels collected at 6-month intervals, despite an intraclass correlation coefficient of 0.64 for salivary progesterone in the same subjects [18]. By contrast, nipple aspirate fluid (NAF) hormone concentrations appear to be substantially more reproducible, as discussed later.

Breast-tissue based risk factors

Other than germ-line mutations that impose an inherited susceptibility to the development of breast cancer, the strongest risk factors for this disease are found locally in the breast (Box 1). These are atypical epithelial proliferation and mammographic density. Local breast hormone levels could potentially fall into this category of breast cancer risk factors, but definitive work in this area is just beginning. We will consider these two factors in relation to what is known about the systemic, and the local breast hormonal environment.

Atypical epithelial proliferation

The present paradigm for the development of breast cancer suggests that in the breast, as in other epithelial organs, the etiologic pathways of malignancy converge in the occurrence of breast epithelial hyperplasia or intra-epithelial neoplasia [19]. This concept is supported by data going back several decades, relating benign breast disease (specifically epithelial proliferation) to an increased risk of subsequent breast cancer. Several studies have shown that the breast cancer risk of women with hyperplasia without atypia is roughly twofold greater than women without, and the risk associated with atypical hyperplasia is increased approximately fourfold [20–23]. The role of local or systemic estrogen levels in driving the appearance of atypia in the breast has not been studied, but can be reasonably postulated since the use of postmenopausal hormone therapy (PMHT) has been shown to increase cellular proliferation in the breast [24].

Mammographic density

The radiographic appearance of the breast varies according to differences in the relative distributions of fat and fibroglandular tissues, where fat appears dark and radiographically dense areas appear light. There is now a substantial body of evidence showing that extensive mammographic breast density is strongly associated with an increased risk of breast cancer. In a recent meta-analysis, the relative risk for the highest category of breast density (>75% of breast area is dense) ranged from 3.25 to 6.49 [25]. The importance of mammographic density as a strong and independent risk factor for breast cancer is amplified by its high prevalence, with approximately a third of the general population of women displaying dense areas of 50% or greater on mammography [25], so that the fraction of breast cancer cases attributable to high mammographic density is in the range of 16 to 32% [26], and higher in premenopausal women [27].

Box 1. Sources of risk factors.

- Many women with breast cancer have no identifiable risk factors. Biomarkers that identify individuals at risk are badly needed.
- Serum hormone levels show only a modest association with breast cancer risk in postmenopausal women.
- Other than germ-line mutations in tumor-suppressor genes, the strongest breast cancer risk factors are found locally in the breast.
- These include atypical epithelial proliferation and mammographic density. Nipple aspiration fluid hormone levels reflect the local hormone content of the breast, and are likely to show stronger associations with breast cancer risk than serum hormone levels.

Breast density reflects (at least partially) the cumulative hormonal exposure of a woman. This is most evident by the fact that the use of combination PMHT increases percent breast density [28,29], and tamoxifen therapy reduces it [30], as does ovarian suppression with a gonadotrophin-releasing hormone agonist in premenopausal women [31]. However, studies examining the correlation between serum sex-steroid levels and mammographic density have not shown any consistent associations, with one large recent study showing no relationship between circulating sex steroid levels and mammographic density [32]. It would be of interest to determine if local breast estradiol levels (e.g., those in NAF) are related more closely to breast density than circulating levels; these studies are ongoing. On the other hand, mammographic density and serum sex steroids (estradiol and testosterone) may have independent and additive effects on breast cancer risk as suggested by a recently reported case-control study nested within the NHS [33]. Hints that mammographic density is modulated by the endocrine environment come from the significant correlation between mammographic density and serum IGF-1 that has been observed in some, but not all studies of premenopausal women [34,35]. Prolactin is another potentially important hormone in breast carcinogenesis; higher levels in the serum are associated with increased breast cancer risk in both pre- and postmenopausal women [36], and several studies now suggest an association between serum prolactin levels and mammographic density [34,37]. Again, NAF levels of IGF-1 and prolactin have not been studied relative to mammographic density, and would be of considerable interest.

Nipple aspiration fluid & breast cancer risk

Composition of NAF

The substances that accumulate in the fluid within the ducts of the mammary gland are, in part, a filtrate of blood (Box 2). This is evidenced

by the presence of a high concentration of proteins, half of which appear to be identical to γ -globulins of blood [38]. In addition, breast fluid contains substances produced by the epithelial and myoepithelial cells as well as by the fibroblasts and other cells in the surrounding tissue. Foam cells are abundant in NAF and they originate apparently from both macrophages and epithelial cells [39]. These macrophages that accumulate in such hypoxic environments may also contribute to soluble substances found in NAF including estradiol, and immunoreactive aromatase in breast tissue macrophages was correlated with lesion severity in one study [40].

The accumulation of steroids in NAF against a concentration gradient from plasma suggests that they are being retained by binding substances in NAF or by cells in the ductal lumen (macrophages) and ductal lining (epithelial cells) with which equilibration occurs with very slow removal. No studies on the presence of binding proteins for steroids in NAF have been conducted to date, but it seems clear that the turnover of substances in NAF does occur, and is slow [41]. The recovery of estradiol concentrations in NAF following 20 days of oral contraceptive (OC) use paralleled to a large degree the increase in serum that occurred within 6 days of OC withdrawal [42]. Whatever the causes of retention of steroids in NAF, the concentrations observed should reflect their availability to cells lining the mammary ducts.

Collection of NAF

The collection of NAF is well tolerated, inexpensive and produces samples that are paucicellular (or acellular) but rich in proteins and hormones. It was first evaluated by Papanicolaou in the 1950s, with the goal of breast cancer detection [43], and was subsequently furthered by Sartorius, who developed a breast pump device designed to improve nipple fluid yield from asymptomatic women [44]. The method in use today involves

Box 2. Risk factors in nipple aspiration fluid.

- The ducts of the non-lactating breast contain fluid that can be expressed at the ductal orifice on the nipple using a combination of breast massage and suction at the nipple (nipple aspirate fluid or nipple aspiration fluid [NAF]).
- The substances that accumulate in the fluid within the ducts of the mammary gland are partly a filtrate of blood.
- NAF also contains substances produced by the epithelial cells and macrophages
- The accumulation of steroids in NAF against a concentration gradient suggests that they are being retained by binding substances in NAF and, in the case of the steroid sulfates, are being actively transported into the ductal lumen.

breast massage, dekeratinization of the nipple and suction aspiration with a commercially available device (Figure 2). NAF ranging from 1 μ l to hundreds of microliters can typically be obtained from 40 to 80% of women with higher success rates in premenopausal women. It is typically collected at the nipple in a calibrated glass capillary tube [45].

Factors influencing NAF production

The ability to obtain NAF depends on several factors [17]. NAF can be obtained most frequently in women who are between the ages of 30 and 60 years, in women of European and African ancestry, and in women from higher socio-economic strata. A history of a young age at menarche, parity and lactation, 'fibrocystic disease' and use of postmenopausal hormone-replacement therapy is associated with the presence of NAF [46]. A more recent analysis of a large cohort study identified four variables that were positively associated with the ability to obtain NAF (being married, history of

pregnancy, tranquilizer use and endocrine disorders). A younger age was consistently associated with a greater ability to obtain NAF in this and other studies [47]. Higher consumption of milk also appears to aid NAF yield in premenopausal women [48]. Factors that do not seem to influence NAF yield include the phase of the menstrual cycle, use of OCs (current or past) and presence of a positive family history of breast cancer.

Hormones in NAF among healthy women

The concentrations of hormones in NAF are generally higher than those in serum [49,50] and are poorly correlated with serum levels. In premenopausal women estradiol was 130–700% greater and progesterone was more than 600% greater in the midluteal phase of the menstrual cycle than the corresponding serum concentrations. The concentrations of estrogens and androgens in extracts of breast tissue also correlate poorly with concentrations in serum, and are similar in breast tissues of pre- and postmenopausal women [51–53]. On the other hand, NAF progesterone was significantly associated with progesterone levels in both serum ($p < 0.001$) and saliva ($p = 0.002$) [49]. No comparison is available between tissue and serum concentrations of progesterone.

Estradiol is clearly synthesized locally by aromatase activity in the pre-adipocytes in the breast [54], and the higher local concentration in tissues of the breast presumably contribute to the concentrations observed in NAF. Sulfates of DHEA and estrone are present in NAF in concentrations many times higher than those in serum and may represent a reservoir of precursors for estradiol, although a small minority of women have undetectable concentrations of both steroid sulfates, with NAF and serum estradiol concentrations in the same range as the other subjects in the study [55,56]. These observations suggest that if estrone sulfate is an important precursor of estradiol in the breast, there must be compensating mechanisms for maintaining estradiol levels in its absence. By contrast, unconjugated DHEA levels in NAF of the subjects with very low or absent steroid sulfates were significantly lower than those with high levels of steroid sulfates (10.2 ± 3.5 vs 56.2 ± 6.7 pmol/l, respectively) indicating that NAF DHEA is a product of DHEAS in the breast.

Progesterone appears to be somewhat unique among the steroids. When blood levels decline, the concentrations in NAF also decline. For

Figure 2. Aspirator for collection of nipple aspirate fluid*.

*Cytoc Corporation, Boxborough, MA, USA.

The plunger on the syringe is withdrawn to the 10 ml mark, for approximately 10 s. This can be repeated several times, with repeat massage and 'stripping' of the nipple.

example, in postmenopausal women, the progesterone concentration of NAF was 5% of that in premenopausal women, while estradiol levels were not significantly less [56]. The high concentrations of progesterone in premenopausal women apparently come from the circulation; there is no evidence for biosynthesis of progesterone in the breast. Nevertheless, a few postmenopausal women have relatively high levels of progesterone in NAF with a discrepancy between breasts: one woman was seen with very high progesterone in the NAF of one breast on two occasions 3 weeks apart while the NAF from her other breast remained below 0.9 ng/ml [55]. Perhaps biosynthesis of progesterone occurs in the breast under some circumstances, and may have implications for breast cancer risk. Testosterone was not found to be significantly associated with breast cancer nor with serum levels of testosterone in one small study [57].

NAF hormones relative to the menstrual cycle, menopause & exogenous hormone use

In a study of NAF hormone fluctuation through the menstrual cycle, ovulation was timed by the subjects using a commercial ovulation kit at home, and salivary estradiol and progesterone were measured daily throughout the cycle [55]. The cyclical fluctuation of hormones was marked in the serum, with significant differences between follicular and luteal phases; fluctuation in NAF was less pronounced, and follicular-luteal differences were not statistically significant (Table 1). This relative blunting of hormone fluctuation in NAF argues for a significant local production of estradiol in the breast, even in premenopausal women.

We have examined the effect of the synthetic sex steroids ethynyl estradiol and 19-nor-progestins present in OCs on NAF hormones in

13 premenopausal women, compared with 47 non-OC users [56]. These women underwent NAF and serum sampling 6 days after completing a cycle of active drug, and then 14 and 21 days later. NAF and serum E2 values were again similar, but much lower with OC use than in the non-OC group, followed by a significant rise in NAF and serum E2 levels 6 days post-withdrawal of OC (Figure 3). These synthetic steroids are known to decrease ovarian production of estradiol and progesterone by threefold and tenfold by reducing gonadotropin secretion, but whether there is any feedback on intramammary aromatase is not known. The impact of combination PMHT on NAF hormones has been studied by comparing six postmenopausal women on hormone therapy with 18 women not on hormone therapy. As expected, hormone users showed higher serum estradiol values than non-users, but NAF estradiol was 18-times higher in hormone users than in control women, whereas serum E2 was only 2.5-fold higher [56]. These findings (Table 2) show that NAF hormone levels reflect the systemic environment, and provide additional support for the idea that NAF hormone content is physiologically relevant. Additionally, the far greater increase in NAF estradiol than in serum estradiol with the use of PMHT raises the possibility that exogenous estrogen may be inducing enzymes such as sulfatase, the expression of which in the breast is known to be induced by estradiol [58].

Stability of NAF hormones over time

The concentrations of NAF hormones and growth factors is relatively consistent within individuals across time, as demonstrated in a study where NAF was collected periodically in the midluteal phase for a period of 15 months (Box 3) [55]. Spearman correlation coefficients were calculated for each of 1000 bootstraps, and

Table 1. Concentrations of estradiol and estrone sulfate in serum and nipple aspirate fluid in ten premenopausal women.

Phase of cycle	Serum			NAF		
	Estradiol (pmol/l)	Estrone sulphate (nmol/l)	Progesterone (nmol/l)	Estradiol (pmol/l)	Estrone sulfate (nmol/l)	Progesterone (nmol/l)
Follicular	169 ± 18	8.4 ± 1.3	1.8 ± 0.4	1200 ± 167	1418 ± 457	1.5 ± 1.0
Luteal	292 ± 36	14.7 ± 2.0	49.2 ± 7.0	1478 ± 324	1971 ± 507	27.7 ± 10.2
p-value	0.0068	0.017	<0.001	0.455	0.427	0.05

Means ± SE (DHS study). DHS: Diet and Hormone Study; NAF: Nipple aspiration fluid. Taken from [50].

Figure 3. Estradiol content in nipple aspirate fluid and serum, during oral contraceptive use and 6 days following withdrawal.

NAF: Nipple aspirate fluid; OC: Oral contraceptive.

Reproduced with permission from [56] © (2004) The Endocrine Society.

correlation coefficients with 95% CIs were averaged across these bootstraps [59,60]. These results were used to examine reproducibility between

breasts at a given time and over time within a single breast. As shown in Table 3, the correlations over a period of 15 months were high, and the correlation in all analytes was higher per breast over time than correlation between breasts. The data are shown graphically in Figure 4. The uniformity of concentration within breasts over time is particularly striking for the steroid sulfates. By contrast, as described in the section on serum hormones, serum hormone reproducibility in premenopausal women is minimal even when the measurements were performed at two time points 1 year apart, on the same day of the menstrual cycle, at the same time of day, after a 12-h fast [17].

NAF hormones & breast cancer risk

The idea that local breast hormone levels are more tightly linked to breast cancer risk than serum hormone levels has obvious appeal, and is acquiring practical importance with the development of therapeutic (and preventive) approaches based on inhibition of extra-ovarian estradiol synthesis, for example, the aromatase and sulfatase inhibitors. With improvements in the understanding of tissue-specific regulation of these enzymes (i.e., breast vs other sites), it may be possible to design pharmacologic interventions, which specifically retard hormone synthesis

Table 2. Geometric mean concentrations of steroids, cathepsin D, EGF and IL-6 in nipple aspiration fluid of normally ovulating premenopausal women, those taking oral contraceptives, as well as postmenopausal women without and with postmenopausal hormone therapy (DHS and SPORE studies).

Analyte	Premenopausal mean concentration (95% CI) n = 47	OC mean concentration (95% CI) n = 13	Postmenopausal mean concentration (95% CI) n = 18	HRT mean concentration (95% CI) n = 7
Estradiol (pmol/l)	316 (271–368)	108 (64–179)*	120 (11–179)	2181 (1660–2868)†
Estrone sulfate (nmol/l)	2000 (1646–2431)	203 (117–346)†	1088 (826–1434)	537 (274–1057)
Progesterone (nmol/l)	132 (111–158)	2.2 (–3.2–5.1)†	6.7 (3.8–12.4)†	4.4 (2.2–8.6)
DHEA (nmol/l)	30.5 (25.3–36.4)	30.2 (19.8–46.4)	40.9 (30.5–54.8)	191 (28–1300)
Androstenedione (nmol/l)	2.1 (1.4–3.0)	1.8 (1.2–2.9)	3.4 (2.7–4.4)	3.6 (1.5–8.6)
DHEA sulfate (μmol/l)	836 (678–1026)	144 (83–249)	809 (589–1107)	298 (152–578)
Cathepsin D (nmol/l)	23.5 (19.5–28.3)	24.0 (12.9–44.5)	24.8 (18.5–33.3)	1.55 (0.85–2.83)†
EGF (nmol/l)	54.0 (49.6–59.0)	34.2 (25.4–45.9)	37.9 (29.5–33.1)	34.0 (23.1–50.1)
IL-6 (pmol/l)	24.6 (20.8–29.6)	47.7 (33.1–69.2)	84.6 (73.8–96.9)†	37.7 (23.5–60.4)

OC and postmenopausal groups were compared with the premenopausal group and HRT was compared with the postmenopausal group.

*Significantly different, $p < 0.05$.

†Highly significantly different, $p < 0.01$, after Bonferroni's adjustment for nine multiple comparisons, respectively.

CI: Confidence interval; DHEA: Dehydroepiandrosterone; DHS: Diet and Hormone Study; HRT: Hormone-replacement therapy;

OC: Oral contraceptive; SPORE: Specialized Programs of Research Excellence (these studies are funded by NCI).

Taken from [56] © (2004) The Endocrine Society.

Box 3. Utility of nipple aspiration fluid as a source of biomarkers.

- Nipple aspiration fluid (NAF) levels of estrogenic hormones are stable over time.
- Precursor hormones that are converted to estradiol correlate well with the estradiol content in NAF, suggesting local synthesis of estradiol in the breast.
- Estrogen-response proteins in NAF have significant correlations with NAF estradiol levels.
- In preliminary studies, NAF estradiol levels are significantly related to breast cancer risk estimates.

in a specific organ. Information regarding local hormone concentration therefore becomes particularly relevant (Box 3).

Interest in nipple-fluid hormone levels dates back to the 1980s, when investigations of NAF hormone levels in relation to breast cancer risk were initiated, but were limited by the immunoassay capabilities of the time, and did not show conclusive results. In a study of women with clinically normal breasts, those with benign disease and those with breast cancer, Ernster *et al.* saw no significant differences in mean values across groups [61]. However, very few women with breast cancer were included in this study. They found that levels in nipple fluid were 5–45-times higher than in the serum, and despite the lower serum estradiol levels in postmenopausal women, the nipple fluid estradiol was not significantly different between pre- and postmenopausal women [52,60]. Similarly, no significant associations were seen between NAF estradiol and various estrogens in serum and saliva in a recent

study [49]. Of interest was the observation that there was a decrease in NAF estradiol for several years after cessation of lactation, and Ernster *et al.* speculated that this finding may explain some of the protective effect of pregnancy and lactation [61]. In a comparison of breast fluid estradiol and estrone levels between nulliparous, parous and lactating women, the same group reported that parous and lactating women had significantly lower levels than those who were nulliparous [62].

Three studies conducted at our institution over recent years have provided insights into the characteristics of NAF hormones in standard-risk versus high-risk women. In the Diet and Hormone Study (DHS; designated group 1 in Figure 5A) 47 healthy, standard risk, premenopausal, regularly cycling volunteers were recruited in the period 1996–1998 with the objective of measuring the concentrations of estradiol, its primary precursors and factors with which it interacts in the breast, and their sources of variation [55]. Their mean age was 35 years. The menstrual cycle was carefully characterized using urinary ovulation detection kits and measurement of salivary hormones daily throughout the menstrual cycle at baseline and at 4, 12 and 15 months. NAF and blood were collected approximately 7 days after ovulation. A second study enrolled 60 healthy standard-risk postmenopausal women during the interval 2001–2003 (group 2 in Figure 5A), with the objective of comparing the patterns of hormones in NAF with those of women taking

Table 3. Mean concentration of analytes in nipple aspirate fluid from premenopausal women with correlations between breasts and over time (DHS study).				
Analyte	Number of subjects (samples)	Concentration (mean ± SE)	Right versus left breast correlations*	Correlations over 15 months per breast*
Protein	47 (271)	74.4 ± 2.1 mg/ml		
Estradiol	31 (188)	435 ± 27 pmol/l	0.61 (0.34–0.78)	0.65 (0.43–0.80)
Estrone sulfate	43 (284)	3.74 ± 0.26 µmol/l	0.84 (0.73–0.91)	0.93 (0.88–0.96)
DHEAS†	45 (292)	75.1 ± 6.7 µmol/l	0.67 (0.47–0.81)	0.88 (0.80–0.93)
DHEA	42 (269)	56.0 ± 6.1 nmol/l	0.65 (0.43–0.79)	0.71 (0.53–0.83)
Androstenedione	24 (147)	6.0 ± 0.6 nmol/l	0.71 (0.47–0.85)	0.77 (0.57–0.88)
Progesterone	43 (288)	291 ± 29 nmol/l	0.71 (0.53–0.83)	0.76 (0.61–0.86)
Cathepsin D	47 (292)	3.42 ± 0.38 µg/ml	0.68 (0.47–0.81)	0.74 (0.58–0.85)
EGF	47 (299)	424 ± 12 ng/ml	0.57 (0.33–0.74)	0.71 (0.54–0.83)
IL-6	45 (291)	1.74 ± 0.21 ng/ml	0.73 (0.53–0.85)	0.64 (0.42–0.79)

*Correlations by the permutation method with 95% confidence interval.
†Measured after hydrolysis as DHEA.
DHEA: Dehydroepiandrosterone; DHEAS: Dehydroepiandrosterone sulfate; DHS: Diet and Hormone Study.
Taken from [55].

Figure 4. Comparison of 15-month and baseline values for eight analytes in nipple aspiration fluid.

Data plots of eight analytes in NAF at BL on the abscissa and at C15 on the ordinate from premenopausal women sampled during the midluteal phase of the menstrual cycle. A perfect correlation would be represented by a diagonal line from zero to the highest point. It is apparent, with the exclusion of the very low values, that there is a good relationship between BL values and C15 values for all analytes, particularly the steroid sulfates.

BL: Baseline; C15: 15 months; DHEA: Dehydroepiandrosterone.

Reproduced with permission from [55].

hormone-replacement therapy and premenopausal women [56]. The third study (group 3 in Figure 5A) was designed to examine ductal lavage as a method of procuring breast epithelial cells from healthy high-risk women [63], but NAF was collected prior to the lavage procedure in a subset of women, and hormone levels were analyzed. The mean age of the women included in the analyses described here was 40 years, and their mean 5-year Gail risk was 3.2. The methods and standards were common to all three studies, with assays performed by the same laboratory [50,55,56].

A pooled analysis of the three aforementioned studies suggests a positive relation between Gail risk and estradiol content of NAF, which appears distinct from the effect of age. As shown in Figure 5A, the NAF estradiol levels of the high-risk-group cluster at a higher level than in the standard-risk women, both pre- and postmenopausal. In analyses stratified by menopausal status, the significant positive correlation between NAF estradiol and Gail risk estimates persisted, whereas the correlation of NAF estradiol with age was not significant [Khan SA, Gann PH, Helenowski IB *et al.*, Unpublished data]. The

three study groups pooled for this analysis came from the same institution and, most importantly, the assays were run in the same laboratory using the same procedures and quality-control standards.

Figure 5. Relationship of Gail risk estimates and nipple aspirate fluid estradiol in three different studies.

(A) Group 1 was from a study of generally low risk premenopausal women who were not recruited based on risk. Group 2 was from a similar group of postmenopausal women. Group 3 was from a group of high risk (5-year Gail estimate >1.6). The relationship is apparent only for Group 3 women who were at higher risk. (B) Quantile double plot of Gail scores and natural log of estradiol in ductal lavage fluid in a high risk (Gail >1.6) group of 36 women (both pre- and postmenopausal). The linear plot demonstrates that the log transformation provides adequate normalization of estradiol to Gail scores for parametric analyses.

However, the characteristics of the three groups of women do differ, with premenopausal standard-risk women, postmenopausal standard-risk women and healthy high-risk women recruited into three studies with distinct time frames. The subjects were pooled in a *post-hoc* analysis, and the data therefore need to be interpreted with caution. However, in a separate study of high-risk women recruited to a study of ductal lavage, we analyzed estradiol and other hormone levels in the lavage fluid. In these samples, the ductal cells were pelleted by centrifugation, the supernatant was lyophilized and reconstituted in a standard volume, and assayed using the same methods as the NAF studies described earlier [Chatterton RT Jr, Avram MJ, Helenowski IB *et al.*: Endocrine profile of breast ductal lavage fluid and its modulation by tamoxifen. Submitted (2008)]. Again, we observed a positive correlation between Gail risk estimates and estradiol content in the lavage supernatant (Figure 5). Given the biological plausibility of this association, and the consistency of the effect across menopausal strata, these data provide the rationale for a careful, prospective examination of the hypothesis that nipple-fluid hormone levels are markers of breast cancer risk.

Proteins in NAF

Growth factors and other proteins are also present in measurable amounts in breast fluid. Of those that are known to be related to estrogen response, several show strong associations with the estradiol content in NAF, suggesting that NAF estradiol is biologically relevant and a measure of the estrogenicity of the local breast environment. For example, EGF, which is known to be induced by estradiol in breast cancer cells [64], and has a clear growth-promoting role in breast cancer [65], was found to relate to the serum levels of estradiol in NAF [59]. A number of other estrogen-response proteins have been measured in breast fluid. Among them are TFF-1 (pS2) and cathepsin D. Harding *et al.* observed that TFF-1 and cathepsin D were higher in the NAF of premenopausal women, increased by hormone-replacement therapy, and were suppressed by the GnRH agonist goserelin and tamoxifen [66]. They also measured two proteins that are suppressed by estrogens, ApoD and gross cystic disease fluid protein 15 (also known as prolactin-inducible protein) and observed the expected decreases in response to the same treatments. Investigators from the same laboratory found that soy supplementation produced increases in TFF-1 and suppression of ApoD in NAF [67].

Basic FGF is also present in NAF and has been shown to be elevated in cancerous breasts and in breasts of women at high risk for breast cancer [68]. The presence of IGF-I has not been reported in breast fluid but its binding protein IGFBP-3 has been shown to be related to the presence of breast cancer in both pre- and postmenopausal women [69]. The levels of IGFBP-3 are inversely related to the presence of the kallikrein prostate-specific antigen (PSA, Hk3), which degrades the binding protein. In the aforementioned study, the association with breast cancer was greater for PSA than for IGFBP-3, although PSA was significantly suppressed only in cancers of premenopausal women. In more recent studies by the same group, four kallikreins have been identified in NAF: hK2, PSA, hK6 and hK10. Of these, PSA appears to display the strongest (albeit negative) association with the presence of breast cancer [70]. Carcinoembryonic antigen (CEA) is also present in NAF in concentrations exceeding 1000 ng/ml but no differences were found between breasts with cancer and those without [71]. Further study of CEA by this group revealed a sensitivity of only 32% for detection of cancer and the CEA concentrations were not different for breasts with proliferative lesions and tumor-free breasts [72].

There is a growing literature on the proteomics profile of NAF; in one study, 41 proteins were identified in NAF [73]. Among those most significantly associated with ductal carcinoma *in situ* were GCDFP15 and ApoD. Continuing proteomic studies of NAF have identified additional proteins, among them a 11.8-kDa protein that purportedly can distinguish between ductal carcinoma *in situ* and invasive disease [74]. Vitamin D-binding protein has been identified in NAF from cancer-bearing breasts in a small study using a different proteomics approach (isotope-coded affinity-tag tandem mass spectrometry), and has been proposed as an early diagnosis marker on this basis [75]. However, protein profiles appear to be similar between cancer-containing and contralateral unaffected breasts, but differ from unaffected healthy women, suggesting that it may be possible to define high-risk profiles [76]. Again, these studies are early in their development and much validation is needed.

Cells in NAF

Nipple fluid contains cells that are exfoliated into the ductal lumen and can be collected at the duct orifice by suction aspiration. The concept of epithelial sampling by nipple aspiration in relation to breast cancer risk was pioneered by Petrakis and

colleagues, who published a series of reports through the 1970s to the present, characterizing nipple-fluid yield and cytologic findings in healthy women. In two cohorts studied by this group, NAF yield varied considerably (85% for the first cohort and 40% for the second) [77,78]. In the first cohort, women who produced NAF and had cytological evidence of hyperplasia developed breast cancer at 2.5-fold higher rate (95% CI: 1.1–5.5), which increased to 4.9-fold (95% CI: 1.7–13.9) when cytologic atypia was present (Figure 3). Women who did not produce NAF were the reference group. In the second cohort, accrued between 1981 and 1991, the RR was 2.0 (95% CI: 1.3–3.3) for the hyperplasia and atypical hyperplasia groups combined, since no cancers occurred in the 22 women with atypical hyperplasia. In general, the cell yield of NAF samples is insufficient to enable large-scale studies that include assessment of biomarkers, and most of the attention devoted to NAF as a window on the breast environment has focused lately on the hormonal and protein components of NAF.

Oxidation markers in NAF

NAF has been analyzed for 8-isoprostane and 2,6-cyclolycopene-1,5-diol as markers of oxidative stress [79]. Levels of 2,6-cyclolycopene-1,5-diol and 8-isoprostane were higher in the NAF than in plasma of normal subjects, but concentrations in the NAF were not correlated with levels in the plasma. The authors suggest that the breast is exposed to high levels of oxidative stress, which cannot be determined from plasma measurements. Further studies indicated that nonsteroidal anti-inflammatory agents could reduce measures of oxidative stress in both plasma and NAF [80]. On the other hand, measurements of malondialdehyde and 8-isoprostaglandin-2 α as indicators of oxidative processes revealed decreases in NAF of breast cancer patients [81]. The utility of such measures of oxidative stress has yet to be determined.

Conclusion

NAF can be collected from 40 to 80% of women less than 60 years of age, and provides an opportunity to measure the hormonal milieu of the breast, and to follow it over time. NAF hormones (with the exception of progesterone) correlate poorly with serum hormones. The hormone content of the breast is more stable than serum, although over a matter of days, NAF levels tend to follow serum levels. Estrogen-response proteins in NAF correlate well with the estradiol content. Early data suggest that women at increased risk of

breast cancer demonstrate higher levels of NAF hormones, supporting the importance of local hormone synthesis in the breast. Research is needed on the processes by which estrogen biosynthesis is increased within the breast so that estrogen concentrations can be predicted from plasma levels of the hormones and other factors that determine local biosynthesis.

Future perspective

Prospective, well-designed research on the constituents of NAF in women with and without breast cancer will lead to the delineation of differences in the hormone and protein profiles of NAF relative to breast cancer risk, and will provide candidate

markers to be pursued in cohort studies. Over the next decade or so, this work is likely to serve as a stepping stone for the identification of molecules that serve the cause of breast cancer risk assessment and prevention, and allow detection of occult tumors that are biologically relevant and likely to progress. The steroid hormones and growth factors clearly have potential as factors that increase the risk of breast cancer. Newer methods based on nanotechnology will provide the sensitivity to measure these hormones in small samples. Such technology will make these tests more feasible in small volumes of NAF or in fine-needle aspirates of the breast. The delineation of regulators of local versus systemic steroid hormone concentrations

Executive summary
Estrogen-related breast cancer risk factors <ul style="list-style-type: none">A number of parameters that reflect the endocrine environment through life are related to breast cancer risk. These include early menarche, late first full-term pregnancy, late menopause, postmenopausal obesity, exogenous hormone use and alcohol use.
Breast tissue-based risk factors <ul style="list-style-type: none">Other than genetic susceptibility through the inheritance of dominant disease-causing mutations, the strongest breast cancer risk factors are found in breast tissue itself. These include atypical epithelial hyperplasia and mammographic breast density.
Nipple aspiration fluid: yield & composition <ul style="list-style-type: none">Nipple aspirate fluid (NAF) is a filtrate of blood, with contributions of hormones and proteins that are locally synthesized. It also contains epithelial and myoepithelial cells, macrophages and fibroblasts. Not all women produce NAF; those who do are likely to be in the age range of 30 to 60 years, premenopausal, married, parous, with a history of lactation.
Nipple aspiration fluid & serum hormones <ul style="list-style-type: none">The steroid hormones concentrations of NAF reflect the local hormonal environment of the breast and are consistent within a breast over periods of a year or more. NAF hormones are therefore potential predictors of breast cancer risk.Correspondence of NAF and serum levels of estrogens is low, as would be expected for a hormone that is synthesized to a significant degree in the breast.Progesterone in NAF corresponds quite well with levels in serum indicating that serum levels are good estimates of exposure of the breast to progesterone.Androgens are present in NAF and provide the substrate that is aromatized to estrogens.Sulfated steroids are present in NAF in concentrations many times greater than in serum and may be a precursor source for estrogen biosynthesis.A number of protein growth factors are present in NAF in concentrations that may provide information on availability to the breast parenchyma. The quantity of estrogen-related proteins in NAF relates well to the estradiol content.
NAF hormones & breast cancer risk <ul style="list-style-type: none">There are so far no robust data relating NAF hormones to breast cancer risk, although preliminary analyses suggest a positive correlation between NAF estradiol and estimated breast cancer risk using the Gail model. A large case-control study is underway. Proteomic analysis of NAF has also yielded some proteins that are present in NAF in higher concentrations in breast cancer patients. However, for both hormones and proteins in NAF, much validation work remains to be done. For large-scale application, NAF components with diagnostic or risk-assessment value would need to be identified in some other compartment (breast cells and serum urine), since not all women produce NAF.

may open up possibilities of organ-specific inhibition of steroid hormone synthesis, thus avoiding the systemic toxicities of systemic therapy while achieving the desired impact of a less estrogenic environment in the breast.

Measurement of biomarker molecules in NAF is unlikely to be useful for population screening, since all women do not produce NAF, and it is unlikely that simple measures will be identified that will increase the fraction of NAF producers to 100%. Eventually, the NAF markers that can be taken to general application for breast cancer risk assessment, or targeting for prevention, or as diagnostic tests for breast cancer screening, will need to be applied to the serum or urine, or even to minimal breast samples obtained by fine-needle aspiration. In this sense, studies of NAF are more likely to serve as a discovery platform, and candidate markers can be validated in

NAF samples, but eventual large-scale application will require that these are also identified in a compartment other than NAF. Markers that are identified through studies of NAF also have the potential of being developed as reagents for molecular imaging, to improve the performance of present imaging techniques.

Financial & competing interests disclosure

The work reported in this review was supported by the Bluhm Family Program for Breast Cancer Early Detection and Prevention, NIH/NCI P50 CA89018-02, R01CA 120555, and the Avon Progress for Patients Program. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

- Vona-Davis L, Howard-McNatt M, Rose DP: Adiposity, Type 2 diabetes and the metabolic syndrome in breast cancer. *Obes. Rev.* 8, 395–408 (2007).
- Friedenreich CM, Orenstein MR: Physical activity and cancer prevention: etiologic evidence and biological mechanisms. *J. Nutr.* 132(Suppl. 11), S3456–S3464 (2002).
- Kendall A, Folkerd EJ, Dowsett M: Influences on circulating oestrogens in postmenopausal women: relationship with breast cancer. *J. Steroid Biochem. Mol. Biol.* 103, 99–109 (2007).
- Gail MH, Brinton LA, Byar DP *et al.*: Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J. Natl Cancer Inst.* 81, 1879–1886 (1989).
- Fisher B, Costantino JP, Wickerham DL *et al.*: Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J. Natl Cancer Inst.* 90, 1371–1388 (1998).
- Rockhill B, Spiegelman D, Byrne C, Hunter DJ, Colditz GA: Validation of the Gail *et al.* model of breast cancer risk prediction and implications for chemoprevention. *J. Natl Cancer Inst.* 93, 358–366 (2001).
- Tyrer J, Duffy SW, Cuzick J: A breast cancer prediction model incorporating familial and personal risk factors. *Stat. Med.* 23, 1111–1130 (2004).
- Kabuto M, Akiba S, Stevens RG, Neriishi K, Land CE: A prospective study of estradiol and breast cancer in Japanese women. *Cancer Epidemiol. Biomarkers Prev.* 9, 575–579 (2000).
- Toniolo PG, Levitz M, Zeleniuch-Jacquotte A *et al.*: A prospective study of endogenous estrogen and breast cancer in postmenopausal women. *J. Natl Cancer Inst.* 87, 190–197 (1995).
- Key T, Appleby P, Barnes I, Reeves G: Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. *J. Natl Cancer Inst.* 94, 606–616 (2002).
- This paper is an important source of evidence consolidating the significance of estradiol and related hormones as biomarkers of breast cancer risk in postmenopausal women.
- Hankinson SE, Willett WC, Manson JE *et al.*: Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women. *J. Natl Cancer Inst.* 90, 1292–1299 (1998).
- Thomas HV, Reeves GK, Key TJ: Endogenous estrogen and postmenopausal breast cancer: a quantitative review. *Cancer Causes Control* 8, 922–928 (1997).
- Tworoger SS, Missmer SA, Barbieri RL, Willett WC, Colditz GA, Hankinson SE: Plasma sex hormone concentrations and subsequent risk of breast cancer among women using postmenopausal hormones. *J. Natl Cancer Inst.* 97, 595–602 (2005).
- Kaaks R, Berrino F, Key T *et al.*: Serum sex steroids in premenopausal women and breast cancer risk within the European Prospective Investigation into Cancer and Nutrition (EPIC). *J. Natl Cancer Inst.* 97, 755–765 (2005).
- Along with [17], illustrates the challenges of studying serum hormones in premenopausal women.
- Page JH, Colditz GA, Rifai N, Barbieri RL, Willett WC, Hankinson SE: Plasma adrenal androgens and risk of breast cancer in premenopausal women. *Cancer Epidemiol. Biomarkers Prev.* 13, 1032–1036 (2004).
- Eliassen AH, Missmer SA, Tworoger SS *et al.*: Endogenous steroid hormone concentrations and risk of breast cancer among premenopausal women. *J. Natl Cancer Inst.* 98, 1406–1415 (2006).
- Muti P, Trevisan M, Micheli A *et al.*: Reliability of serum hormones in premenopausal and postmenopausal women over a one-year period. *Cancer Epidemiol. Biomarkers Prev.* 5, 917–922 (1996).
- Along with [14], illustrates the challenges of studying serum hormones in premenopausal women.
- Chatterton RT Jr, Mateo ET, Hou N *et al.*: Characteristics of salivary profiles of oestradiol and progesterone in premenopausal women. *J. Endocrinol.* 186(1), 77–84 (2005).
- Shaughnessy JA, Kelloff GJ, Gordon GB *et al.*: Treatment and prevention of intraepithelial neoplasia: an important target for accelerated new agent development: recommendations of the American Association for Cancer Research Task Force on the Treatment and Prevention of Intraepithelial Neoplasia. *Clin. Cancer Res.* 8, 314–346 (2002).

20. Dupont WD, Page DL: Risk factors for breast cancer in women with proliferative breast disease. *N. Engl. J. Med.* 312, 146–151 (1985).
21. Carter CL, Corle DK, Micozzi MS, Schatzkin A, Taylor PR: A Prospective study of the development of breast cancer in 16,692 women with benign breast disease. *Am. J. Epidemiol.* 123(3), 467–477 (1988).
22. London SJ, Connolly JL, Schnitt SJ, Colditz GA: A prospective study of benign breast disease and the risk of breast cancer. *JAMA* 267, 941–944 (1992).
23. Hartmann LC, Sellers TA, Frost MH *et al.*: Benign breast disease and the risk of breast cancer. *N. Engl. J. Med.* 353, 229–237 (2005).
24. Hargreaves DF, Knox F, Swindell R, Potten CS, Bundred NJ: Epithelial proliferation and hormone receptor status in the normal post-menopausal breast and the effects of hormone replacement therapy. *Br. J. Cancer* 78, 945–949 (1998).
25. McCormack VA, dos Santos Silva SS: I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. *Cancer Epidemiol. Biomarkers Prev.* 15, 1159–1169 (2006).
26. Byrne C, Schairer C, Wolfe J *et al.*: Mammographic features and breast cancer risk: effects with time, age, and menopause status. *J. Natl Cancer Inst.* 87, 1622–1629 (1995).
27. Boyd NF, Guo H, Martin LJ *et al.*: Mammographic density and the risk and detection of breast cancer. *N. Engl. J. Med.* 356, 227–236 (2007).
28. Greendale GA, Reboussin BA, Sie A *et al.*: Effects of estrogen and estrogen-progestin on mammographic parenchymal density. Postmenopausal Estrogen/Progestin Interventions (PEPI) Investigators. *Ann. Intern. Med.* 130, 262–269 (1999).
29. Rutter CM, Mandelson MT, Laya MB, Seger DJ, Taplin S: Changes in breast density associated with initiation, discontinuation, and continuing use of hormone replacement therapy. *JAMA* 285, 171–176 (2001).
30. Cuzick J, Warwick J, Pinney E, Warren RM, Duffy SW: Tamoxifen and breast density in women at increased risk of breast cancer. *J. Natl Cancer Inst.* 96, 621–628 (2004).
31. Spicer DV, Ursin G, Parisky YR *et al.*: Changes in mammographic densities induced by a hormonal contraceptive designed to reduce breast cancer risk. *J. Natl Cancer Inst.* 86, 431–436 (1994).
32. Verheus M, Peeters PH, van Noord PA, van der Schouw YT, Grobbee DE, van Gils CH: No relationship between circulating levels of sex steroids and mammographic breast density: the Prospect-EPIC cohort. *Breast Cancer Res.* 9, R53 (2007).
33. Tamimi RM, Byrne C, Colditz GA, Hankinson SE: Endogenous hormone levels, mammographic density, and subsequent risk of breast cancer in postmenopausal women. *J. Natl Cancer Inst.* 99, 1178–1187 (2007).
34. Boyd NF, Stone J, Martin LJ *et al.*: The association of breast mitogens with mammographic densities. *Br. J. Cancer* 87, 876–882 (2002).
35. Bremnes Y, Ursin G, Bjurstam N, Rinaldi S, Kaaks R, Gram IT: Endogenous sex hormones, prolactin and mammographic density in postmenopausal Norwegian women. *Int. J. Cancer* 121, 2506–2511 (2007).
36. Tworoger SS, Eliassen AH, Sluss P, Hankinson SE: A prospective study of plasma prolactin concentrations and risk of premenopausal and postmenopausal breast cancer. *J. Clin. Oncol.* 25, 1482–1488 (2007).
37. Greendale GA, Huang MH, Ursin G *et al.*: Serum prolactin levels are positively associated with mammographic density in postmenopausal women. *Breast Cancer Res. Treat.* 105, 337–346 (2007).
38. Vorherr H: Human lactation and breast feeding. In: *Lactation: A Comprehensive Treatise*. Larson BL (Ed.). Academic Press, New York, NY, USA 181–280 (1978).
39. Damiani S, Cattani MG, Buonamici L, Eusebi V: Mammary foam cells. Characterization by immunohistochemistry and *in situ* hybridization. *Virchows Arch.* 432, 433–440 (1998).
40. Mor G, Yue W, Santen RJ *et al.*: Macrophages, estrogen and the microenvironment of breast cancer. *J. Steroid Biochem. Mol. Biol.* 67, 403–411 (1998).
41. Miller WR, Hawkins RA, Creel RJ, Forrest AP: Oestrogen in breast fluid. *Lancet* 2, 1179 (1977).
42. Chatterton RT, Geiger AS, Khan SA: Influence of ovarian secretions, oral contraceptives, and hormone replacement therapy on progesterone concentrations in breast fluids. *Breast Cancer Res.* 82(2), S110 (2004).
43. Papanicolaou GN, Holmquist DG, Bader GM, Falk EA: Exfoliative cytology of the human mammary gland and its value in the diagnosis of cancer and other diseases of the breast. *Cancer* 11(2), 377–409 (1958).
44. Sartorius OW, Smith HS, Morris P, Benedict D, Friesen L: Cytologic evaluation of breast fluid in the detection of breast disease. *J. Natl Cancer Inst.* 59, 1073–1080 (1977).
45. Sauter ER, Ross E, Daly M *et al.*: Nipple aspirate fluid: a promising non-invasive method to identify cellular markers of breast cancer risk. *Br. J. Cancer* 76, 494–501 (1997).
46. Wrensch MR, Petrakis NL, Gruenke LD *et al.*: Factors associated with obtaining nipple aspirate fluid: analysis of (1428) women and literature review. *Breast Cancer Res. Treat* 15, 39–51 (1990).
47. Baltzell KA, Wrensch M, Sison JD: A descriptive study of variables associated with obtaining nipple aspirate fluid in a cohort of non-lactating women. *BMC Womens Health* 6, 15 (2006).
- **Comprehensive discussion of the determinants of nipple aspiration fluid (NAF) yield in a large cohort by an experienced group of investigators.**
48. Huang Y, Anderson KE, Nagamani M, Grady JJ, Lu LJ: Dietary intake of lactose as a strong predictor for secretor status of nipple aspirate fluid in healthy premenopausal nonlactating women. *Clin. Cancer Res.* 14, 1386–1392 (2008).
49. 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.* 15, 39–44 (2006).
- **Provides good evidence that NAF hormone concentrations are biologically relevant, since they correlate well with the quantities of estrogen-response proteins in NAF.**
50. 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.* 86, 159–166 (2003).
51. Thijssen JH, van Landeghem AA, Poortman J: Uptake and concentration of steroid hormones in mammary tissues. *Ann. NY Acad. Sci.* 464, 106–116 (1986).
52. Blankenstein MA, Szymczak J, Daroszewski J, Milewicz A, Thijssen JH: Estrogens in plasma and fatty tissue from breast cancer patients and women undergoing surgery for non-oncological reasons. *Gynecol. Endocrinol.* 6, 13–17 (1992).

53. Geisler J: Breast cancer tissue estrogens and their manipulation with aromatase inhibitors and inactivators. *J. Steroid Biochem. Mol. Biol.* 86, 245–253 (2003).
54. Bulun SE, Sharda G, Rink J, Sharma S, Simpson ER: Distribution of aromatase P450 transcripts and adipose fibroblasts in the human breast. *J. Clin. Endocrinol. Metab.* 81, 1273–1277 (1996).
55. Chatterton RT Jr, Geiger AS, Khan SA, Helenowski IB, Jovanovic BD, Gann PH: Variation in estradiol, estradiol precursors, and estrogen-related products in nipple aspirate fluid from normal premenopausal women. *Cancer Epidemiol. Biomarkers Prev.* 13, 928–935 (2004).
- Detailed analysis of the reproducibility of NAF hormones over a 15-month period, with four repeated measures in cycling women.
56. 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.* 90, 1686–1691 (2005).
57. Sauter ER, Tichansky DS, Chervoneva I, Diamandis EP: Circulating testosterone and prostate-specific antigen in nipple aspirate fluid and tissue are associated with breast cancer. *Environ. Health Perspect.* 110, 241–246 (2002).
58. Zaichuk T, Ivancic D, Scholtens D, Schiller C, Khan SA: Tissue-specific transcripts of human steroid sulfatase are under control of estrogen signaling pathways in breast carcinoma. *J. Steroid Biochem. Mol. Biol.* 105, 76–84 (2007).
59. Gann P, Chatterton R, Vogelsong K, Dupuis J, Ellman A: Mitogenic growth factors in breast fluid obtained from healthy women: evaluation of biological and extraneous sources of variability. *Cancer Epidemiol. Biomarkers Prev.* 6, 421–428 (1997).
60. Good P: *Permutation Test: A Practical Guide to Resampling Methods for Testing Hypotheses (3rd Edition)*. Springer-Verlag, New York, NY, USA (2004).
61. Ernster VL, Wrensch MR, Petrakis NL *et al.*: Benign and malignant breast disease: initial study results of serum and breast fluid analyses of endogenous estrogens. *J. Natl Cancer Inst.* 79, 949–960 (1987).
62. Petrakis NL, Wrensch MR, Ernster VL *et al.*: Influence of pregnancy and lactation on serum and breast fluid estrogen levels: Implications for breast cancer risk. *Int. J. Cancer* 40, 587–591 (1987).
63. Bhandare D, Nayar R, Bryk M *et al.*: Endocrine biomarkers in ductal lavage samples from women at high risk for breast cancer. *Cancer Epidemiol. Biomarkers Prev.* 14, 2620–2627 (2005).
64. Dickson RB, Huff KK, Spencer EM, Lippman ME: Induction of epidermal growth factor related polypeptides by 17 β -estradiol in MCF-7 human breast cancer cells. *Endocrinology* 118, 138–142 (1986).
65. Kim H, Muller WJ: The role of the epidermal growth factor receptor family in mammary tumorigenesis and metastasis. *Exp. Cell. Res.* 253, 78–87 (1999).
66. Harding C, Osundeko O, Tetlow L, Faragher EB, Howell A, Bundred NJ: Hormonally-regulated proteins in breast secretions are markers of target organ sensitivity. *Br. J. Cancer* 82, 354–360 (2000).
67. Hargreaves DF, Potten CS, Harding C *et al.*: Two-week dietary soy supplementation has an estrogenic effect on normal premenopausal breast. *J. Clin. Endocrinol. Metab.* 84, 4017–4024 (1999).
68. Sartippour MR, Zhang L, Lu M, Wang H-J, Brooks MN: Nipple fluid basic fibroblast growth factor in patients with breast cancer. *Cancer Epidemiol. Biomarkers Prev.* 14, 2995–2998 (2005).
69. Sauter ER, Chervoneva I, Diamandis A, Khosravi JM, Litwin S, Diamandis EP: Prostate-specific antigen and insulin-like growth factor binding protein-3 in nipple aspirate fluid are associated with breast cancer. *Cancer Detect. Prev.* 26, 149–157 (2002).
70. Sauter ER, Klein G, Wagner-Mann C, Diamandis EP: Prostate-specific antigen expression in nipple aspirate fluid is associated with advanced breast cancer. *Cancer Detect. Prev.* 28, 27–31 (2004).
71. Foretova L, Garber JE, Sadowsky NL *et al.*: Carcinoembryonic antigen in breast nipple aspirate fluid. *Cancer Epidemiol. Biomarkers Prev.* 7, 195–198 (1998).
72. Zhao Y, Verselis SJ, Klar N *et al.*: Nipple fluid carcinoembryonic antigen and prostate-specific antigen in cancer-bearing and tumor-free breasts. *J. Clin. Oncol.* 19, 1462–1467 (2001).
73. Alexander H, Stegner AL, Wagner-Mann C, Du Bois GC, Alexander S, Sauter ER: Proteomic analysis to identify breast cancer biomarkers in nipple aspirate fluid. *Clin. Cancer Res.* 10(22), 7500–7510 (2004).
74. Sauter ER, Shan S, Hewett JE, Speckman P, Du Bois GC: Proteomic analysis of nipple aspirate fluid using SELDI-TOF-MS. *Int. J. Cancer* 114, 791–796 (2005).
75. Pawlik TM, Hawke DH, Liu Y *et al.*: Proteomic analysis of nipple aspirate fluid from women with early-stage breast cancer using isotope-coded affinity tags and tandem mass spectrometry reveals differential expression of vitamin D binding protein. *BMC Cancer* 6, 68 (2006).
76. Pawlik TM, Fritsche H, Coombes KR *et al.*: Significant differences in nipple aspirate fluid protein expression between healthy women and those with breast cancer demonstrated by time-of-flight mass spectrometry. *Breast Cancer Res. Treat.* 89, 149–157 (2005).
77. Wrensch MR, Petrakis NL, King EB *et al.*: Breast cancer incidence in women with abnormal cytology in nipple aspirates of breast fluid. *Am. J. Epidemiol.* 135, 130–141 (1992).
78. Wrensch MR, Petrakis NL, Miike R *et al.*: Breast cancer risk in women with abnormal cytology in nipple aspirates of breast fluid. *J. Natl Cancer Inst.* 93, 1791–1798 (2001).
79. Chen G, Djuric Z: Detection of 2,6-cyclolycopene-1,5-diol in breast nipple aspirate fluids and plasma: a potential marker of oxidative stress. *Cancer Epidemiol. Biomarkers Prev.* 11, 1592–1596 (2002).
80. Kato I, Chen G, Djuric Z: Non-steroidal anti-inflammatory drug (NSAID) use and levels of a lipid oxidation marker in plasma and nipple aspirate fluids. *Breast Cancer Res. Treat.* 97, 145–148 (2006).
81. Mannello F, Tonti GA, Pagliarini S *et al.*: The 8-epimer of prostaglandin F(2 α), a marker of lipid peroxidation and oxidative stress, is decreased in the nipple aspirate fluid of women with breast cancer. *Int. J. Cancer* 120, 1971–1976 (2007).