The local hormonal environment and related biomarkers in the normal breast

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Abstract

Recent developments in breast epithelial sampling techniques (nipple fluid aspiration, ductal lavage, and random fine needle aspiration) provide new opportunities for the acquisition of hormonal and cellular biomarker data in asymptomatic women, and thereby the possibility of developing a unified vision of how the hormonal environment of the breast may interact with the cellular expression of proteins, and with other evolving candidate markers of breast cancer risk. The purpose of this review is to integrate available information regarding cellular and breast fluid biomarkers of hormone action on the breast, to identify candidate biomarkers for studies of breast cancer risk and prevention. These include the estrogen receptors α and β , markers of proliferative and apoptotic response, and protein markers of estrogen action in breast cells and nipple fluid. Studies of breast hormone levels in nipple aspiration fluid (NAF) show that estrone sulphate is present in large quantities in the normal breast, while the differences in serum ovarian steroids that are seen in pre- and postmenopausal women are blunted in NAF. The variability of several estradiol precursors in NAF over time is relatively small, a useful attribute of potential biomarkers of breast cancer risk, particularly if they are reversible with intervention in Phase 2 prevention trials. These studies are already providing new insights into the hormonal etiology of breast cancer, and should lead to the identification of robust, reversible biomarkers for use in breast cancer prevention studies.

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Introduction

The past decade has seen a proliferation of information regarding the endocrinology and intracrinology of the normal breast, and parallel information regarding the expression of hormone-related biomarker expression in the breast. However, relatively few attempts have been made to synthesize these two areas into an understanding of how local hormonal metabolism influences potential biomarkers such as proliferation, apoptosis and hormone receptor expression. With the availability of breast epithelial and ductal fluid sampling techniques such as nipple fluid aspiration, ductal lavage (DL) and random fine needle aspiration (rFNA), there is now an opportunity to measure both the hormone levels of breast ductal fluid, and the cellular response in the form of proliferation, apoptosis and the expression of estrogen-response

proteins. This review addresses the available literature on cellular biomarkers of hormone-related breast cancer risk in breast tissue samples, and developing areas using newer sampling techniques, which promise to increase our understanding of normal breast biology, and point to candidate biomarkers for use in prevention studies.

The current paradigm

The normal non-lactating breast is subject to cyclical changes in response to ovarian stimulation with either estrogen alone during anovulatory cycles, or estrogen plus progesterone during ovulatory cycles. These hormones increase breast epithelial cell proliferation, with progesterone having a synergistic/additive effect during ovulatory cycles. The withdrawal of hormones

with the onset of a new ovarian cycle causes a wave of apoptosis, eliminating defective cells and maintaining the population balance of the epithelium. Repeated waves of proliferation, however, lead to an accumulation of genetic defects, with the eventual emergence of a transformed cell, leading to the clonal evolution of malignancy (Cohen & Ellwein 1991, King 1993, Pike *et al.* 1993). Therefore, factors that increase availability of hormones, augment the proliferative response of the epithelium to hormonal stimulation, or interfere with apoptotic elimination of defective cells, will increase breast cancer risk. Factors which decrease hormone availability, blunt proliferative response, or aid apoptotic elimination will protect against breast cancer.

This paradigm is consistent with present evidence: length of reproductive life and number of ovulatory cycles increase breast cancer risk (Hankinson & Hunter 2002) and breast epithelial proliferation is highest in the luteal phase (Ferguson & Anderson 1981). Exposure to high postmenopausal serum estrogen levels contributes to increased risk of breast cancer (Trichopoulos *et al.* 1983, Hankinson *et al.* 1998). However, there are gaps; no long-term studies have related high proliferative rates of breast epithelium to increased risk of breast cancer. The value of proliferation as a surrogate endpoint in the breast has not been demonstrated, and the role of apoptosis (whether increased or decreased in the high-risk breast) is poorly understood. The importance of local breast hormone levels with regard to breast cancer risk has not been studied.

Studies of serum hormone levels and hormone metabolism relevant to breast cancer

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 have been observed in almost all of the larger studies, with relative risk estimates ranging from 1 to 4 for several of the estrogenic hormones studied. In particular, the results of a nested case-control analysis of the Nurses Health Cohort showed the strongest effects for estrone sulfate (ES), dehydroepiandrosterone sulfate (DHEAS) and estradiol, with relative risks of 4.34, 4.15 and 3.53 respectively (Hankinson *et al.* 1998). Several other case-control studies have shown that serum estradiol

approached significant trends within quartiles or quintiles (Toniolo *et al.* 1995, Kabuto *et al.* 2000). An overview analysis of studies published until 1997 concluded that serum estradiol levels in women who develop breast cancer are about 15% higher than in those who do not (Thomas *et al.* 1997).

Among premenopausal women, serum hormone levels were measured at specified intervals during the menstrual cycle in a case-control study of 169 breast cancer subjects; no significant relationships were found with estradiol in any part of the menstrual cycle (Sturgeon *et al.* 2004). A possible solution to the problem of fluctuating hormone levels in premenopausal women is the measurement of estradiol and progesterone in salivary samples, since saliva can be collected by the subjects at home and stored without refrigeration for subsequent analysis once a full month of samples has been collected. The data can be analyzed to give an integrated level over the entire menstrual cycle. In addition, salivary steroids are thought to reflect levels of unbound, bioavailable serum steroids, and may be more relevant to studies of breast hormonal exposure than serum or plasma steroid levels.

Measurements of hormones in nipple aspiration fluid (NAF)

With the development of therapeutic approaches based on inhibition of extra-ovarian estradiol synthesis, and data regarding local synthesis of estrogen in breast tumors, there is increasing interest in the idea that local hormone levels in the breast may have more relevance to promotion of breast cancer than serum hormone levels. The analysis of estrogens in NAF has been performed by several investigators in the past (Rose *et al.* 1986, Ernster *et al.* 1987, Petrakis *et al.* 1987), and we have performed several studies of hormones and hormone response proteins in the soluble fraction of NAF (Chatterton *et al.* 2000, 2003, 2004, 2005). The results from these studies are discussed below.

Relationship of hormones in NAF to concentrations of the hormones in serum

In several studies of serum and NAF estradiol and progesterone, the correlations between serum and NAF hormone levels have been variable. Our first and smallest study ($n = 10$) showed a correlation between serum and NAF estradiol of 0.55, with a mean mid-luteal phase serum estradiol of 292 ± 36 pmol/l and simultaneous NAF estradiol of 1478 ± 324 pmol/l, (Chatterton *et al.* 2003). However, subsequent studies, using the same methodology have not shown a

Table 1 Summary of literature on serum and NAF hormone levels

Reference (number subjects)	Estradiol (pmol/L $\pm$ SE)		Estrone (pmol/L $\pm$ SE)	
	Serum	NAF	Serum	NAF
Ernster <i>et al.</i> 1987				
Premenopausal (76)	354	3166	284	4822
Postmenopausal (11)	56	3101	123	4433
Petrakis <i>et al.</i> 1987				
Never pregnant (23)	378	536	281	5276
Gravid-nulliparous (20)	362	4598	275	6574
Parous (36)	433	1832	323	2838
Rose <i>et al.</i> 1986				
Premenopausal (24)	323 $\pm$ 70	2882 $\pm$ 727	240 $\pm$ 63	3722 $\pm$ 912
Chatterton* <i>et al.</i> 2003				
	Estradiol (pmol/L)		Estrone sulfate (nmol/L)	
	Serum	NAF	Serum	NAF
Follicular (10)	169 $\pm$ 18	1200 $\pm$ 167		1418 $\pm$ 457
Luteal (10)	292 $\pm$ 36	1478 $\pm$ 324		1971 $\pm$ 507
Chatterton* <i>et al.</i> 2005				
Premenopausal (47)	328 (304–352)	316 (271–368)	12.8 (11.2–14.5)	2000 (1646–2431)
OC users (13)	94.7 (74.9–120.1)	108 (64–179)	2.7 (2.3–3.2)	203 (117–346)
Postmenopausal (18)	52.5 (46.3–59.5)	120 (11–179)	1.8 (1.6–2.1)	1088 (826–1434)
HRT users (7)	131 (86.6–198.3)	2181 (1660–2868)	5.3 (3.5–8.2)	537 (274–1057)

*Estradiol levels in NAF were assayed after extraction and purification. The purification procedure, solvent partition between 0.4 N NaOH and isooctane, resulted in values 8-fold lower than those obtained after extraction alone (Chatterton *et al.* 2004). Data of other investigators were obtained without purification. Reproduced from Chatterton *et al.* 2004 with permission from *Cancer Epidemiology, Biomarkers & Prevention*.

significant correlation between serum and NAF estradiol. In a second study of 47 women the serum estradiol concentration at mid-luteal phase was similar (356 ± 65.4 pmol/l), whereas in NAF it was lower (484 ± 28 pmol/l) (Chatterton *et al.* 2004). The latter value corresponds closely to concentrations of estradiol in tissue of premenopausal women when the tissue concentration is expressed in pmol/g (Thijssen *et al.* 1986, Blankenstein *et al.* 1999). There was no correlation between concentrations of DHEAS and ES in serum and those in NAF. Correlations between serum and NAF hormone levels have not generally been published in older studies, although Ernster *et al.* (1987) reported a correlation of 0.1 between serum and NAF estradiol, and 0.14 between serum and NAF estrone, both of which were not statistically significant.

Progesterone, which has not been measured previously in NAF, averaged 207.2 ± 3.8 ng/ml in our first study, and serum averaged 15.5 ± 2.2 ng/ml. In the second study NAF progesterone averaged 91.5 ± 9.9 ng/ml and serum averaged 15.5 ± 0.3 ng/ml. The correlations between serum and NAF in the two studies were 0.27 and 0.20 respectively. Thus, accumulation of progesterone relative to serum levels was also significantly lower in the second group of subjects,

who were also more physically active, suggesting an effect of exercise on uptake or retention of estrogen and progesterone by the breast.

In a subsequent study, using similar methods, we have examined the effects of oral contraceptives (OCs) and hormone replacement therapy (HRT) on NAF and serum hormone levels (Chatterton *et al.* 2005). The correlations between serum and NAF hormones are generally poor but mean NAF hormone levels reflected the overall trends in mean hormone levels seen in the serum. Thus, estradiol levels were lower in both serum and NAF of OC users compared with controls, and higher in serum and NAF of HRT users compared with controls. However, in postmenopausal HRT users, the differences in NAF estradiol were more marked than those in serum estradiol: NAF estradiol of women on HRT was 18-fold higher than postmenopausal control women, whereas serum estradiol was only 2.5-fold higher. From these data, it appears that changes in serum hormones are reflected in NAF, but only after a matter of days.

Our results are in general agreement with the older literature in this area (summarized in Table 1) in that NAF hormone levels are higher than serum levels, and the decline in serum estradiol seen with menopause is

not seen to the same extent in NAF (Chatterton *et al.* 2005). There have been few investigations of NAF hormone levels in relation to breast cancer risk. In a study of women with clinically normal breasts, those with benign disease, and those with breast cancer, Ernster *et al.* (1987) saw no significant differences in mean values across groups. Petrakis *et al.* (1987) have compared the breast fluid estradiol and estrone levels between nulliparous, parous and lactating women, and have reported that parous and lactating women had significantly lower levels than those who were nulliparous. And Rose *et al.* (1986) have compared NAF hormone levels in American and Finnish women, and found those in Finnish women (who have a lower breast cancer risk) to be lower. Previously reported estradiol levels in NAF, measured without purification, were 2 to 8 times higher than those reported by Chatterton *et al.* 2003, 2004 (Rose *et al.* 1986, Ernster *et al.* 1987). This difference was shown to be potentially attributable to cross-reactivity of the antiserum with ES and DHEAS, which are present in 10^4 and 10^5 times greater concentrations respectively than estradiol in NAF (Chatterton *et al.* 2004). Purification of the samples was required for elimination of the cross-reacting steroids.

Relationship between estradiol precursors and estradiol in NAF

Data from the second group described above include measurement of potential androgen precursors that may be metabolized to estradiol within the breast. With estradiol as the outcome measure, and accounting for the other variables such as breast and time of sampling by covariance techniques, the β -estimate for DHEA was 0.153 ± 0.060 ($P = 0.017$) (Chatterton *et al.* 2004). Thus, even in premenopausal subjects androgen precursors were significantly related to the levels of estradiol in the breast.

ES is of interest because of its potential as a precursor of estradiol and its high concentration in NAF. ES within the breast was also related to NAF estradiol; the β -estimate was 0.201 ± 0.066 ($P = 0.004$). Since ES circulates in relatively high concentration, and is concentrated in breast fluid, it may also be an important source of estradiol in the breast, as proposed by others (Pasqualini *et al.* 1996, 1997, Purohit *et al.* 1999).

We have found evidence of conversion of ES to estrone in breast parenchymal cells from grossly normal breast tissue of women undergoing excision of benign lumps (Chatterton *et al.* 2003). Despite the low concentration of ES in the cells, it was converted

linearly within a 1000-fold concentration range of up to $10 \mu\text{M}$ to estrone by intact cells, having a mean half-time of conversion of $628 \text{ min}/10^6$ cells. Upon comparing the rate of metabolism of ES in homogenized cells to that in intact cells, we found that homogenized cells had a conversion half-time that was about 50% shorter, suggesting that the plasma membrane is a barrier to conversion to estrone. These results are consistent with the reported site of the sulfatase in the microsomal fraction of the cells (Hernandez-Guzman *et al.* 2001). However, it is also possible that hydrolysis of ES occurs without entering the cell. Willemssen *et al.* (1988) have shown that estrogen sulfatase activity also resides on the plasma membrane. In this case, the high levels of estrone sulfatase that enter the lumen of the ductal tree, apparently by an active transport process (Pizzagalli *et al.* 2003), may be hydrolyzed to estrone before entering the epithelial cells. Thus estrogen sulfatase activity would make ES a ready source of estrone to the mammary parenchyma.

Relationship between estrogens in breast fluid and estrogen response proteins

Estradiol promotes the synthesis of both epidermal growth factor (EGF) and cathepsin D; both are present in measurable concentrations in NAF, and may indicate whether the concentrations of estradiol in NAF are biologically meaningful. In the second group of 47 subjects mentioned above, both estradiol and ES were significantly related to NAF EGF levels; β -estimates were 0.082 ± 0.035 ($P = 0.024$) and 0.216 ± 0.035 ($P = 0.0001$) respectively (Chatterton *et al.* 2004). Similarly, cathepsin D showed a significant relationship to estradiol and ES, with β -estimates of 0.28 ($P = 0.015$) and 0.32 ($P = 0.002$). Similar estimates of the relationships between serum estradiol and EGF and cathepsin D were not statistically significant in our study. NAF pS2 has also been shown to increase in postmenopausal women starting HRT, and decrease in premenopausal women starting goserelin therapy (Harding *et al.* 2000). Overall, it seems reasonable to assume that NAF estrogen concentration provides a better estimate of biologically active estrogen in the breast than do serum levels of estradiol. The fact that ES had higher correlations with these products may relate to the fact that its concentration is very high in NAF and easily and accurately measured. On the other hand, levels of estradiol in NAF were often at the lower limit of sensitivity of the method and for approximately 30% of the samples were not detected by the immunoassay method.

Table 2 Mean concentration of analytes in nipple aspirate fluid from premenopausal women with correlations between breasts and over time

Analyte	Number of Subjects (Samples)	Concentration (mean $\pm$ SE)	Right vs Left Breast Correlations*	Correlations over 15 months per breast*
Protein	47 (271)	74.4 $\pm$ 2.1 mg/ml		
Estradiol	31 (188)	435 $\pm$ 27 pmol/L	0.61 (0.34, 0.78)	0.65 (0.43, 0.80)
Estrone sulfate	43 (284)	3.74 $\pm$ 0.26 μ mol/L	0.84 (0.73, 0.91)	0.93 (0.88, 0.96)
DHEAS**	45 (292)	75.1 $\pm$ 6.7 μ mol/L	0.67 (0.47, 0.81)	0.88 (0.80, 0.93)
DHEA	42 (269)	56.0 $\pm$ 6.1 nmol/L	0.65 (0.43, 0.79)	0.71 (0.53, 0.83)
Androstenedione	24 (147)	6.0 $\pm$ 0.6 nmol/L	0.71 (0.47, 0.85)	0.77 (0.57, 0.88)
Progesterone	43 (288)	291 $\pm$ 29 nmol/L	0.71 (0.53, 0.83)	0.76 (0.61, 0.86)
Cathepsin D	47 (292)	3.42 $\pm$ 0.38 μ g/ml	0.68 (0.47, 0.81)	0.74 (0.58, 0.85)
EGF	47 (299)	424 $\pm$ 12 ng/mg	0.57 (0.33, 0.74)	0.71 (0.54, 0.83)
IL-6	45 (291)	1.74 $\pm$ 0.21 ng/ml	0.73 (0.53, 0.85)	0.64 (0.42, 0.79)

*Correlations by the permutation method with 95% CI.

**Measured after hydrolysis as DHEA.

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Variability of NAF hormones over time

In the group of 47 premenopausal women described above, we determined the stability of hormone measurements in NAF over a 15 month period. Saliva was collected daily throughout the baseline menstrual cycle and at 4, 12 and 15 months to ascertain menstrual cycle characteristics, and NAF and blood were collected approximately 7 days after ovulation. The analytic methods have been described previously (Chatterton *et al.* 2004). Concentrations of these analytes were compared between breasts of each woman and within breasts across the full 15 month period. The data are listed in Table 2, showing that the correlation over time is very good, and slightly greater than the correlation of simultaneous measurements between breasts.

The baseline and 15 month measurements are plotted against each other in Fig. 1. The concentrations of DHEAS and ES are particularly stable with correlations within women across 15 months of 0.93 and 0.88 respectively. This stability, which may be attributed in part to the precision of measurement of the high concentrations of these analytes found in NAF, is encouraging in terms of the potential utility of these measures as surrogate endpoints for interventions which seek to lower local hormone levels in the breast. However, it is not clear at the moment whether or not sulfated estrone and DHEA in the breast are activated by steroid sulfatase, and to what extent they engender an estrogenic response in the breast epithelium. While NAF estradiol is in the same range or a few fold higher than concentrations in blood, the concentrations of DHEAS and ES are many-fold higher than

blood levels. These concentrations cannot be attributed to high-affinity binding substances in NAF, and the high concentration appears to be limited to the breast fluid and not the epithelial cells (Chatterton *et al.* 2003). An anion transporter has been described which may be involved in the very high concentrations of steroid sulfates in breast fluid (Pizzagalli *et al.* 2003).

Studies of kallikreins in NAF and relationship to hormones

Sauter *et al.* (1996) have investigated the role of human kallikrein-3 (hK-3), also known as prostate specific antigen (PSA) in the breast as a marker for breast cancer risk. In a study of breast cancer patients and control women, an inverse relationship was seen between NAF PSA and the presence of epithelial proliferation and malignancy, particularly after the menopause. NAF PSA levels were associated with the mid-cycle rise of serum progesterone in seven of eight women but not with serum estradiol or gonadotropins, indicating that sampling must be done at the same stage of the menstrual cycle for consistent results (Sauter *et al.* 1998). A negative association was seen between NAF PSA and disease progression from Duct carcinoma *in situ* to metastatic breast cancer (Sauter *et al.* 2004a,b). Lower PSA concentrations have also been described in estrogen- and progesterone-receptor-negative tumors (Black *et al.* 2000). Additional hK-2, -6 and -10 are expressed in the breast, but hK-6 and hK-10 were not associated with breast cancer. hK-2 exhibited patterns similar to that of pS2 but did not provide additional useful information (Sauter *et al.* 2004b).

Figure 1 Distribution of log values for each analyte. Log values of the concentrations of each analyte in samples collected in the 15th month (C15) are plotted against the corresponding log values in the baseline (BL) samples. A correlation of 1.0 would be represented by a diagonal line from the lower left corner to the upper right of each plot. Plots of values from the 4th and 12th months vs the baseline samples are not shown for brevity, but the distributions were similar to those shown for the 15th month. That is, there was little increase in dispersion between the 4th and 15th months. Reproduced from Chatterton *et al.* 2004 with permission from *Cancer Epidemiology, Biomarkers & Prevention*.

Estrogen receptors in the normal breast

Breast tissue studies

Estrogen receptor- α (ER α) occupies a pivotal position in breast cancer biology, and has been the subject of intense study in malignant tumors. The earliest studies, using ligand-binding assays, showed little ER α

expression in the normal breast (Feherty *et al.* 1971, Wittliff *et al.* 1971), but subsequent immunohistochemical (IHC) methods showed that a relatively small proportion (under 10%) of luminal epithelial cells express ER α in the normal breast, whereas myoepithelial and stromal cells do not (Petersen *et al.* 1987). A number of IHC studies then followed, and established

that ER α expression is higher in the follicular than in the luteal phase of the menstrual cycle (Battersby *et al.* 1992), and that expression is higher in European than in non-European women (Ricketts *et al.* 1991). Since steroid receptor content appears to limit cellular response to steroids (Vanderbilt *et al.* 1987, Webb *et al.* 1992), we hypothesized that increased ER α expression in histologically normal breast epithelium may result in an augmented response to available estrogen, and may be a risk factor for breast cancer. In an initial study of 120 women, ER α expression in benign breast epithelium was measured using an IHC assay (Abbott ER-ICA) on frozen sections. ER α -positive epithelium was found in a greater proportion of cases than controls (85 vs 55%), with an adjusted odds ratio (OR) of 6.5, 95% confidence interval (CI) 1.4–28.9 (Khan *et al.* 1994). This study was extended in order to examine the influence of breast cancer risk factors such as age at menarche, parity and menopause, and use of exogenous estrogens on breast epithelial ER α expression (Khan *et al.* 1999). Again, women with breast cancer expressed higher levels of ER α than benign disease controls, but this was independent of known breast cancer risk factors, with the exception of alcohol use, where there was a trend towards higher ER α expression in premenopausal women who consumed more than three alcoholic drinks per week. We also observed that the breast epithelium of controls on HRT displayed significantly higher ER expression than that of controls not on HRT, an observation subsequently reproduced by Lawson *et al.* (2001), who showed that obesity also contributes to this higher expression.

The notion of increased ER α expression in the high-risk breast is supported by the consistency of high expression over time, demonstrated in a study of 28 women undergoing two separate breast biopsies at least 6 months apart (Khan *et al.* 2002). On categorizing the women into high and low expressers based on an ER labeling index (ER LI) value of 10, there was 86% concordance between the first and second samples. Among premenopausal women, the ability to predict the second ER LI from the first was improved significantly by the addition of the adjusted day of the menstrual cycle at the time of sampling, suggesting that a substantial part of the inter-sample variation in premenopausal women was explained by variation in the menstrual cycle day.

Ethnic variations suggest that groups at higher risk for breast cancer also have higher levels of ER expression: in a study comparing ER α expression in Australian women of European origin to that in Japanese women living in Japan, ER α expression was

higher in the former, consistent with their higher breast cancer risk (Lawson *et al.* 1999). The same group of investigators then compared ER α expression in Japanese women in Hawaii with those in Japan, and found non-significantly higher levels in the Hawaiian Japanese, again in agreement with their higher breast cancer risk (Lawson *et al.* 2002). Finally, there are now several studies documenting the increase in the proportion of ER α -expressing cells seen in proliferative breast lesions (Jacquemier *et al.* 1982, Shoker *et al.* 1999, 2000).

Evidence of dysregulation of ER α expression relative to the hormonal environment

Analysis of ER α expression by menstrual phase in our case-control study (Khan *et al.* 1998) revealed that the proportion of positive cells declined in luteal phase in controls, as seen in studies of naturally cycling women (Battersby *et al.* 1992). However, in women with cancer, this luteal phase decrease was not observed. There was a significant case-control difference in the ER LI in the 25–28 day interval ($P < 0.03$), and a significant trend towards increasing ORs through the menstrual cycle, from an OR of 1.3 in days 1–5, compared with an OR of 4.4 in days 25–28 ($P < 0.015$). These data suggest that ER α expression varies differently between cases and controls through the menstrual cycle. Since other lines of evidence suggest that an increase in ambient estradiol leads to down-regulation of the receptor (Saceda *et al.* 1988, Ree *et al.* 1989), we then looked at ER α expression in normal breast epithelium relative to circulating estradiol levels. Here we found a significant negative correlation between serum estradiol and breast epithelial ER LI in the controls, but no correlation in women with breast cancer (Khan *et al.* 1999). These findings together suggest a disturbance of hormonal regulation of ER α expression in the breast epithelium of high-risk women.

Do ER α -expressing cells proliferate?

Studies of dual labeling of breast epithelial sections for ER α and markers of proliferation have suggested that ER α -expressing cells are distinct from proliferating cells, since markers of proliferation (Ki-67, [3 H]thymidine) do not co-localize with ER α (Clarke *et al.* 1997, Russo *et al.* 1999). Clarke *et al.* (1997) have suggested that ER α -expressing cells are a distinct population and exert a paracrine influence on surrounding cells, which respond by proliferating. As the normal breast progresses through increasing degrees of neoplasia, the separation of the two compartments

blurs, and co-localization of proliferation-related antigens and ER α is seen, suggesting a dysregulation of ER α expression. However, the number of co-localizing cells remains very small in malignancies, increasing from 0.03% in normal epithelium, to 1.3% in invasive cancer (Clarke *et al.* 1997). The alternative explanation (that ER α expression is a cell cycle-related phenomenon) is addressed in a recent study of wild-type and ER β $-/-$ mice (Cheng *et al.* 2004). Oophorectomized mice were exposed to estradiol, an ER β agonist, tamoxifen, or placebo. Bromodeoxyuridine (BrdU) was infused for labeling of S-phase cells, and mice were killed at 2 or 48 h. This study confirmed that estradiol down-regulates ER α in mammary epithelial cells; co-localization of ER α and BrdU was not observed at 2 h, but was seen at 48 h, suggesting that ER α -expressing cells do go on to divide, and do not represent two distinct cell populations.

ER α data from studies using minimal sampling techniques

More recent studies using methods such as rFNA also suggest that increased expression of ER α correlates with the probability of breast cancer development. In a comparison of 244 high-risk and 30 low-risk women, Fabian *et al.* (1997) found that ER α expression was significantly related to the presence of cytological atypia, and the subsequent development of cancer, particularly if a low threshold of positivity (1+) was used. These findings are consistent with our breast tissue studies, where the ability to discriminate cases from controls based on ER α expression in normal and non-proliferative epithelium is optimal with a low threshold of ER α positivity (Khan *et al.* 1998). In a subsequent analysis of 480 women studied by rFNA, the same group found that ER α expression greater than 1+ was the only biomarker among a set of five (DNA ploidy, p53, EGF receptor, HER2/NEU, ER α) that was significantly associated with the subsequent development of malignancy, although in the final multivariate model, only Gail risk and cytological atypia remained significant (Fabian *et al.* 2000).

In DL samples from high-risk women, we have encountered much higher levels of ER α positivity than we reported previously in the control groups of our earlier studies, with a median LI of 26%, probably because we are using newer, more sensitive antibodies, and are studying high-risk women who may have higher proportions of ER-positive cells. So far, we have seen reassuring stability of ER expression over two time points 6 months apart, when the second sample is obtained in the same phase of the menstrual

cycle as the first (Bhandare *et al.* 2003). Our on-going study will also provide information as to whether or not high ER α expression in benign or atypical epithelium identifies women more likely to benefit from endocrine prevention strategies.

ER β

The second estrogen receptor, ER β , was cloned in 1996, and has been intensively investigated regarding its role in normal mammary gland development and breast cancer (Kuiper *et al.* 1996, Gustafsson & Warner 2000). Its relevance as a marker of risk, or as a surrogate endpoint in prevention studies is not clear as yet, but deserves investigation for several reasons, including the fact that it appears to influence activity of ER α . Several studies on normal breast epithelium have revealed that ER β is more abundant than ER α in the nuclei of epithelial cells lining the ducts and the lobules, and that a strong staining pattern is seen in myoepithelial cells, with varying intensity of staining documented in intralobular stromal cell nuclei, nuclei of endothelial cells and occasional lymphocytes (Speirs *et al.* 2002). ER β is expressed in the majority of normal lobules, in contrast to ER α (median fraction of positive cells over 90% in one study, compared with 22.6% for ER α (Shaaban *et al.* 2003)). The proportion of positive cells decreases with increasing degrees of neoplasia, with a steady decrease in proliferative lesions, carcinoma *in situ*, and invasive disease (Roger *et al.* 2001). Thus the ratio of ER β :ER α measured at the mRNA level decreases from normal breast to breast cancer (Leygue *et al.* 1998).

Relative levels of ER α and ER β are an important determinant of cellular sensitivity to estrogens. Although ER α is the stronger transcriptional activator of the two, at physiological concentrations of estradiol, coexpression of ER β results in suppression of both the efficacy and potency of hormone-stimulated responses. The ability of ER β to function as a transcriptional activator or inhibitor, depending on the agonist concentration, suggests that completely different patterns of gene expression may be observed at different hormone levels (Hall & McDonnell 1999). Such interactions may influence the action of potential preventive agents such as genistein (one of the major phytoestrogens in soy), which appears to have a greater affinity for ER β than for ER α (Kuiper *et al.* 1997). This could indicate that phytoestrogens work as antioxidants and that ER β is involved in the control of antioxidant-regulated genes, the products of which control the concentrations of free radicals and reactive oxygen species in the cell (Gustafsson 1999).

Proliferation and apoptosis in normal breast

Normal breast epithelial proliferation has been extensively studied relative to the menstrual cycle, with general agreement that a rise in proliferative index occurs in the luteal phase (Meyer 1977, Anderson *et al.* 1982, Potten *et al.* 1988). This has been particularly well illustrated by studies of FNA in spontaneously cycling women who underwent FNA in the follicular and luteal phase, with ovulation being assessed by the measurement of serum progesterone (Soderqvist *et al.* 1997). A significant correlation was seen between serum progesterone values and Ki-67 labeling, but none with serum estradiol, testosterone, androstenedione, or several peptide hormones. The use of OCs appears to increase breast epithelial proliferation (Anderson *et al.* 1989), a fact which needs to be reconciled with the fairly minimal impact of OC use on breast cancer risk (Westhoff 1999). In postmenopausal women, a significant relationship has been observed between the use of HRT and breast epithelial proliferation. In a study analyzing FNA samples prior to, and 3 and 6 months following the initiation of HRT in 45 postmenopausal women, Conner *et al.* (2003) found that the percentage of Ki-67-positive cells increased more than 4-fold after just 3 months of HRT. There was a positive correlation between percentage of Ki-67-positive breast cells and circulating levels of both estradiol and estrone after 3 and 6 months of treatment. In contrast, no increase in the proliferation index was seen in a cross-sectional study by Hargreaves *et al.* (1998) where biopsy material from women on HRT was compared with those not using it. These findings serve to emphasize the point that the variability of these measurements between individuals is significant, and that longitudinal, within-person comparisons are significantly more sensitive than cross-sectional study designs.

However, prospective evidence that cell proliferation indices in the normal epithelium and benign breast lesions may serve as a marker of breast cancer risk is still lacking, and there are no longitudinal studies demonstrating that the proliferative index of normal epithelium or proliferative lesions relates to breast cancer risk. Such studies are difficult to perform, since the proliferative index of breast epithelium is low, reproducible quantitation of proliferative indices in large numbers of samples is problematic, and in premenopausal women samples need to be acquired in a specific menstrual phase. These barriers have not so far been overcome in human studies. However, one study of ER α expression and proliferative indices of

hyperplastic lesions in women who subsequently developed breast cancer, compared with those who did not, suggests that the Ki-67 labeling index (Ki-67 LI) is higher in the hyperplastic foci of women who later developed cancer (Shaaban *et al.* 2002). In this small study (27 cases and 16 controls), the cases and controls were not age-matched and although both ER α and Ki-67 are known to vary significantly with age in normal epithelium, the relationship to age in hyperplastic lesions has not been assessed. The OR was higher for ER α LI (2.3, 95% CI 0.7–7.2) than for Ki-67 LI (1.5, 95% CI 0.5–5.8).

The measurement of proliferative activity as a potential surrogate endpoint biomarker in the assessment of preventive efficacy of new agents needs similar validation in long-term studies. Several studies of short-term neoadjuvant therapy in women with breast cancer have shown a significant decrease in the proliferative indices of malignant lesions with endocrine therapy, but this decrease was not independently predictive of response (Chang *et al.* 2000). In another study of 32 women without known breast cancer who were given letrozole treatment, estradiol levels dropped significantly, but there was no significant change in proliferative indices at 3 months (Harper-Wynne *et al.* 2002). The problems of utilizing Ki-67 labeling as a predictor of response are illustrated in a subsequent non-randomized study by the same group, where Ki-67 LI was examined as a predictor of response in 106 women with large breast cancers who were treated with tamoxifen, chemotherapy or a combination (Assersohn *et al.* 2003). Another group of 37 untreated women underwent two separate FNAs to assess the reproducibility of Ki-67 labeling; analysis of their proliferative indices suggested that a minimal relative decline of 39% was required for statistical significance. For the final analysis, the authors excluded women whose Ki-67 LI was under 5% because of concerns regarding the precision of the measurement; this resulted in 20 of 33 (61%) of the tamoxifen-treated group being excluded. In the chemoprevention setting then, where baseline proliferative indices tend to run around 2%, the difficulties of utilizing Ki-67 labeling as a surrogate biomarker seems significant. Data from ongoing studies should help resolve these questions.

Published data on proliferative indices in cytological samples of breast epithelium obtained by DL are not available so far, but again the hurdles discussed above with regard to the variability of measuring low frequency events would be compounded by the small samples available in such studies. We have found that the Ki-67 LI of DL samples has a median value of

0.3%, measured in a median of 5690 cells. Whether or not this value is lower than that reported in FNA studies because exfoliated cells tend not to be proliferating, or for other reasons, is not clear at the moment, but trypan blue exclusion rates of these samples range from 50 to 90% (Khan *et al.*, unpublished observations).

Apoptosis in normal breast epithelium

Apoptosis or programmed cell death plays a critical role in normal mammary gland development. Homeostasis in normal tissue is a balance of proliferation and apoptosis, and alteration in this balance has been postulated to set off a series of changes ultimately leading to malignancy (Thompson 1995). Morphologically, apoptotic cells exhibit characteristic changes such as cell contraction, cytoplasmic blebbing, nuclear condensation and genomic autodigestion into nucleosomal fragments. The measurement of apoptosis in the normal breast has been performed by the counting of apoptotic bodies on histological sections, by the TUNEL (TdT-mediated dUTP nick-end labeling) reaction, and more recently by IHC detection of activated caspase-3, which represents the final enzymatic event leading to DNA fragmentation (Gown & Willingham 2002). There is agreement that a wave of apoptosis occurs in late luteal phase (Anderson *et al.* 1982, Williams *et al.* 1991), and that proliferative and apoptotic rates increase as epithelium progresses from normality to malignancy (Allan *et al.* 1992, Bai *et al.* 2001). Two studies of apoptosis and proliferation in normal breast epithelium from women with breast cancer and those undergoing benign breast biopsy for conditions such as fibroadenoma concluded that the ratio of proliferative to apoptotic indices is higher in women with breast cancer (Allan *et al.* 1992, Hassan & Walker 1998), suggesting that decreased elimination of epithelial cells may contribute to the development of malignancy. However, as pointed out by Anderson (1999) in a review of the subject, parity, lactation, contraceptive use, and age of the two groups were not controlled for, and all of these factors are known to influence cell kinetics in the normal breast. In addition, the method of measurement of apoptosis deserves some comment. Allan *et al.* used classic morphological criteria, whereas Hassan & Walker used a combination of *in situ* end-labeling and morphology. The apoptotic indices reported by these authors vary by an order of magnitude (0.1 to 1.2), compared with 0.3 reported in another study where morphology alone was used to identify apoptotic cells (Potten *et al.* 1988). Studies

using TUNEL report values ranging from 0.0 (Bai *et al.* 2001) to 0.04 (Wong *et al.* 2001), to 2.1 per terminal duct lobular unit (Feuerhake *et al.* 2003). Using TUNEL, we have found values of 0.2% for premenopausal women, and 0.1% for postmenopausal women. Although we found no significant differences in median apoptosis and proliferative indices in breast cancer cases as opposed to benign disease controls, a multivariate model including TUNEL and Ki-67 LI, and menopausal status, yielded an OR of 3.66 (95% CI 1.22–10.96) for the TUNEL index (Khan & Stickle 2000). Another study of normal breast epithelium and usual ductal hyperplasia on women with long-term follow-up also showed no predictive value of these measures for the subsequent development of malignancy, although ratios were not calculated (Mommers *et al.* 2001). Thus, although there has been widespread recognition that the study of apoptosis in the normal breast and pre-malignant lesions is important, normal breast studies have been hampered by methodological discrepancies, and as in studies of proliferation, the quantitation of apoptosis in large numbers of samples remains a problem today. The fact that apoptotic rates in normal epithelium and benign lesions are even lower than proliferative rates is another barrier to the inclusion of this parameter as a surrogate endpoint biomarker in studies of risk and prevention.

Inter-relationship between hormone response parameters in cytological samples

The ability to measure breast hormone levels in NAF and duct lavage fluid, and relate these to the cellular parameters discussed above provides an opportunity to test the paradigm presented at the beginning of this review. Based on this paradigm, we would expect a hierarchical relationship between estrogenic hormones, the fraction of ER α -positive cells, Ki-67 labeling, epithelial cell number and cytological atypia. We are presently conducting a study of high-risk women, where the DL effluent is separated into cells and supernatant, allowing analysis of both breast hormone levels and cellular parameters in the same samples. So far, we have been able to observe fragments of this hierarchy: hormonal parameters (particularly ES and DHEAS) relate significantly to cell number, and to cytological atypia; cell number and atypia are strongly inter-related, but the intermediary link with Ki-67 labeling is so far not evident. These women will be re-studied after 6 months on tamoxifen therapy, and the same parameters re-examined, providing new data on the effects of tamoxifen on the endocrine environment and hormone responsiveness of the high-risk and normal breast.

Conclusions

Of the estrogen-related endpoints that have been studied so far, the largest amount of data exists regarding ER α expression and proliferation. The former is an established therapeutic target, and may be of utility as a marker of risk and for selecting women for endocrine prevention strategies. The variability of the latter, at least as measured by Ki-67 labeling, is a significant concern and may ultimately prove to be a barrier to its utilization as a surrogate endpoint in prevention studies. The variability of apoptosis on repeat sampling has not been established, but the low proportion of apoptotic cells in breast epithelial samples, and the difficulties of quantitation related to this, makes it an unlikely surrogate endpoint. Additional studies are needed to establish the optimal menstrual cycle phase for breast epithelial sampling in premenopausal women, the variability of potential surrogate endpoints on repeat sampling in untreated women, and the variability of measurement of specific endpoints, particularly those that are present in small proportions of epithelial cells, such as proliferation and apoptosis. Additional work also needs to be done on the utility of nipple and duct fluid hormone concentrations, their reproducibility in untreated individuals, and their relationship to breast cancer risk. If validated, these are attractive candidates for intermediate biomarkers, particularly in studies of agents which modify local hormone synthesis in the breast.

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