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ORIGINAL PAPER

Expression of Estrogen Receptor Alpha and Beta in Breast Cancers of Pre- and Post-menopausal Women

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Abstract Expression of estrogen receptors (ER) is clinically relevant in designing therapeutic strategies. The relative importance of the two types of estrogen receptors (ER-alpha and ER-beta) in human breast cancers in pre- and post-menopausal women has not been properly defined. To determine the possible association between the expression of estrogen receptor and serum estradiol levels in pre- and post-menopausal women with breast cancer. 44 patients with invasive ductal carcinoma of the breast were studied and a breast tissue biopsy was taken. ER-alpha and ER-beta were detected by immunocytochemistry. Serum levels of estradiol and estrone were measured by radioimmunoassay and FSH was measured using IRMA. We studied 21 pre- and 23 post-menopausal women with breast carcinoma. Examining the number of cases with tumors positive for ER, we found no differences in the frequency of ER-alpha between pre- and post-menopausal women, but ER-beta decreased marginally after menopause ($p<0.051$). In cases with tumors positive for ER, the proportion of cells positive for ER-alpha was similar post-menopausally (53.95%) and pre-menopausally (57.21%), but for ER-beta the number of positive cells decreased significantly after menopause ($p<0.051$). In pre-menopausal women there was a correlation between serum estradiol levels and ER-beta; in post-menopausal women there was a correlation between serum FSH levels and ER-alpha. These results indicate that estradiol levels in women with mammary carcinoma are related to ER-beta expression in the breast tumor tissue.

Keywords Estrogen receptor · Estradiol · Breast cancer

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Background

Estrogens exert different effects, mainly on proliferation and cellular differentiation, through specific nuclear receptors in such diverse organs as hypothalamus, pituitary gland, uterus, breast, ovary, bone and liver [1]. Estrogen receptor (ER)-alpha (α) was cloned and sequenced from human mammary carcinoma (MCF-7) [2]; a second molecular form, ER-beta (β), was identified from a gene bank expression of human testis [3]. Other variants have been identified [4,5]. Both isoforms can coexist in the same tissue and in the same cells, forming functional heterodimers [6]. ERs transduce the hormonal signal, and the hormone-receptor complex activates a wide range of genes that regulate a cascade of secondary events [7]. ER-α is a predictive marker for breast cancer and is used to establish whether adjuvant anti-estrogen treatment is indicated. In contrast, the function of ER-β in breast pathobiology is unclear, partly because most studies have focused on its mRNA rather than the protein [8].

There is evidence that chronic exposure to high estrogen levels is an important risk factor for mammary carcinoma [6]. Estrogen exposure is variable during menopause. Obesity may be associated with significant endogenous estrogen exposure; some women with body mass index (BMI) > 30 produce more estrogen, as suggested by our previous finding of low FSH levels in obese post-menopausal women [11]. Quantification of estrogen receptors in tissues might be useful for estimating actual tissue sensitivity and for inferring previous estrogen exposure.

Menopausal women experience physiological and biological changes, which are explained by different tissue sensitivities to estrogen [9,10]. Nevertheless, circulating estradiol levels are not the only determinants of ER expression, since expression of the two molecular forms in different tissues in pre- and post-menopausal women is controversial. Meza et al. [11] reported that ER and progesterone receptor (PR) decrease abruptly after menopause. At that stage the number of receptors expressed depends on such factors as BMI, years since menopause and serum levels of androgen precursors. Recent investigations of the imbalance between the two molecular forms of ER showed lower expression of ER-β than of ER-α in many cancers, including breast, ovary, colon and prostate, but not in normal tissues [12].

Some clinical and in vitro studies suggest that imbalance in ER-α/ER-β expression is common and important in the progression of hormone-dependent tumors. Lazennec et al. and Bardini et al. [12, 13] obtained the first evidence that ER-β is an important modulator of proliferation and invasion by breast and ovarian cancer cells, supporting the hypothesis that loss of ER-β expression could be one event leading to the development of breast and ovarian cancers. ER-β plays a key role in the mitogenic action of estrogens and is functionally antagonistic to ER-α, which induces hyperproliferation [13]. In this report, ER-β expression is related to the response to hormone treatment [14]. Nevertheless, in most studies, ER expression in post-menopausal women has been analyzed without distinguishing between the two isoforms. It seems necessary to evaluate the effects of hormones on the expression of both estrogen receptors in breast cancer. In this study, we have used immunocytochemistry to investigate the effects of hormones on ER-α and ER-β expression in primary breast tumors in pre- and post-menopausal women.

Patients and Methods

We evaluated 21 pre- and 23 post-menopausal women with breast carcinoma. The pre-menopausal women were aged 27–47 years and had normal regular cycles. The post-menopausal women were older than 48 years; at least 1 year had elapsed since the last menses and the cycles had previously been regular. Inclusion criteria were: no hysterectomy, oophorectomy, diabetes, smoking habit or hormone therapy during the previous six months.

Tumor samples were collected from consecutive breast carcinoma patients in the Department of Oncology, and from the Rodolfo Padilla Foundation and the National Cancer Institute of Mexico. Informed consent for the study was obtained from all patients. Forty-four ductal breast carcinoma samples were obtained using tru-cut biopsies. The results were validated on the surgical specimens. Body mass index (BMI) was calculated using weight and height. Fasting blood samples were obtained for determination of hormone levels. Blood samples from the pre-menopausal women were taken on days 7–14 of the menstrual cycle. Serum levels of estradiol and estrone were measured by radioimmunoassay using the coat-a-count procedure (Diagnostic Products Corporation, Los Angeles, CA, USA). FSH was measured using IRMA (Diagnostic System Laboratories Corporation, Weister, TX, USA).

Histopathology and Immunohistochemistry

Tissue samples were fixed in buffered formalin and paraffin-embedded. Sections were cut at 5–6 μm and placed on slides previously covered with DL-lysine. One slide was stained with hematoxylin–eosin for analysis and classification by two independent pathologists, using three-tiered nuclear and histological grading in accordance with the Bloom and Richardson grading system. The other (unstained) sections were de-paraffinized and used for immunohistochemistry. Epitopes were retrieved by autoclaving in 10 mM citrate buffer, pH 6.1, for 15 min. Slides were incubated overnight with the following primary antibodies: F-10 anti-human monoclonal ER-α (Santa Cruz Biotechnology, 1:100) and H-150 anti-human polyclonal ER-β (Santa Cruz Biotechnology, 1:100) in 1% bovine serum albumin-phosphate buffered saline (BSA-PBS). These antibodies against ERs have been used successfully to distinguish the two molecular forms in breast cancer [15]. Immunostaining was carried out using the Universal DAKO LSAB2 system according to the manufacturer's instructions. Alternate sections were processed by replacing the primary antibody with commercial mouse IgG cocktail (N-Universal Negative Control, DAKO N-1698 Carpinteria, CA) and rabbit IgG cocktail (N-Universal Negative Control, DAKO N-1699) to provide negative controls. Immunoreactivity was estimated by the Allred scores of 100 malignant cells to calculate the percentage of positive cells in each set. Immunostained slides were scored as previously described. First, a proportion score was assigned, which represented the estimated proportion of

Table 1 Clinical-pathological data of breast cancer cases

Clinical parameter	Pre-menopausal women n=21	Post-menopausal women n=23	p
Age (years)	40.2±6.5	57.4±8.6	0.02[a]
BMI (kg/m^2)	27.6±4.7	27.8±3.6	Ns[a]
Hormonal levels			
Estradiol (pg/ml)	177.7±54.53	22.3±6.39	0.0001[a]
FSH (UI/L)	46.36±12.96	58.3±5.6	0.009[a]
Estrone (pg/ml)	75.0 4±35.72	25.4±4.8	0.11[a]
Clinical stage			
II	5	9	Ns[b]
III and IV	16	14	Ns[b]
Axillary node status			
Positive	8	11	Ns[b]
Negative	13	12	Ns[b]
Histologic grade			
1 and 2	5	7	Ns[b]
3	16	16	Ns[b]

[a] Italic figures indicate that the comparison was carried out using the Mann–Whitney *U*-test.
[b] Fisher's exact test (two-tailed).The p value significant≤0.05.

positively-stained tumor cells (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; 5, >2/3). Then an intensity score was assigned, representing the average intensity of the positive tumor cells (0, none; 1, weak, 2, intermediate; 3, strong). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8 [16].

Table 2 Cases with positive of ERβ and ERα according to menopausal status

	Pre-menopausal n=21	Post-menopausal n=23	P
ERα	18 (85.7%)	21 (91.30%)	<0.12
ERβ	11 (52.3%)	6 (26.08%)	<0.051*

Cases with positive ER in breast tumor tissue
*Fisher's exact test (two-tailed).The P value significant≤0.05.

Statistical Analysis

All variables were tested for normality; when significant departure from normality was found, the data were log transformed. We compared the proportions and intensities of both molecular forms of ER in both groups using a *t*-test for independent samples. When significant departure from normality was found, a Mann–Whitney *U*-test was carried out. Factors associated with the amounts of ER-α and ER-β were analyzed using a stepwise multiple regression procedure for both groups and for pre- and post-menopausal women separately. As candidate regressors, BMI, age, and FSH, estradiol and estrone levels were tested. For the post-menopausal group, time (years) since diagnosis was also included. Bonferroni's correction for multiple comparisons was calculated on the P values. For analysis we used Statistica Version 5.0 (StatSoft Inc., Tulsa, OK). Significance was accepted at $p<0.05$.

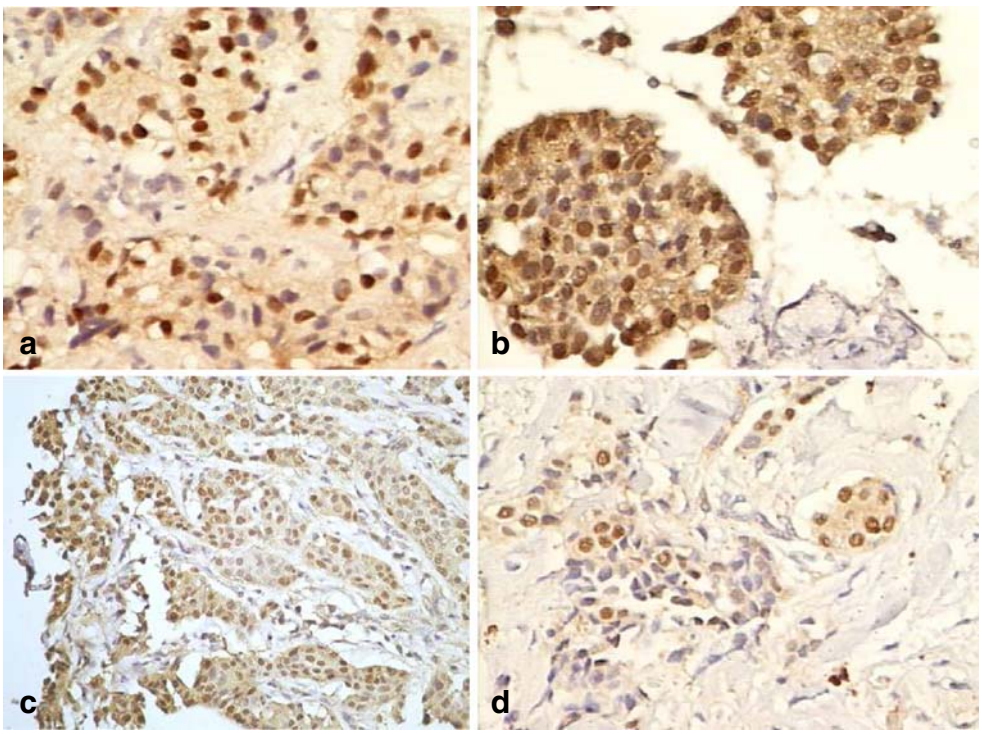

Fig. 1 **a**,**b** Infiltrating ductal carcinoma showing nuclear staining for ERα. **c** ERα positive tumor sourrounded by negative connective tissue. **d** ERβ positive tumor

Table 3 Proportion of positive cells in the cases considered as positive for ER and score Allred

	Pre-menopausal $n=21$		Post-menopausal $n=23$		p
	%	Score IH	%	Score IH	
ERα	53.95±40.58	5.42±2.61	57.21±39.77	5.69±2.61	$t=3.4$ $p<0.45$
ERβ	43.95±45.29	3.80±3.76*	18.78±35.98	1.73±3.07*	$t=0.75$ $p<0.001$

*The p value significant≤0.05.

Results

Patient Characteristics

The clinical characteristics and hormone levels of the 44 patients included in the study are shown in Table 1. Of these patients, 47.72% were pre-menopausal and 52.27% post-menopausal. The median ages were 40.23±6.57 years for the pre-menopausal patients and 57.47±8.69 years for the post-menopausal women. Of the post-menopausal patients, 30.4% had previously used HRT for a mean duration of 6 years.

Immunohistochemistry

Positive staining for ER-α and ER-β (brown DAB deposits) was mostly found in the nuclei of malignant cells. Some immunostaining was also seen in the cytoplasm of the same cells. Surrounding cells in the stroma such as fibroblasts, macrophages, lymphocytes and endothelial cells were unstained. Normal breast tissue showed scattered staining in the duct epithelium for ER-α and only occasional nuclear staining for ER-β (Fig. 1).

Expression of ER-β and ER-α in the whole group of patients

The number of cases positive for ER-β and ER-α differed between the two groups ($p=0.0001$). The immunoreactivity of ER-α expressed as percentage of positive cells was 64.44±5.70%; the corresponding value for ER-β was 78.43±6.38% ($t=2.8$, $p=0.005$). The Allred score and the percentages are shown in Tables 2 and 3.

Association of Menopausal Status with the Expression of ER-β and ER-α

The number of cases positive for ER-β and ER-α was compared between the pre- and post-menopausal women (Figs. 2 and 3). We found no differences in the frequency of ER-α-positive cells (Tables 2 and 3), but there was a slight decrease in ER-β ($p<0.051$). The percentage of ER-α-positive cells was similar before and after menopause ($p<0.45$), but for ER-β the number of cells was significantly lower after menopause ($p<0.001$; Tables 2 and 3).

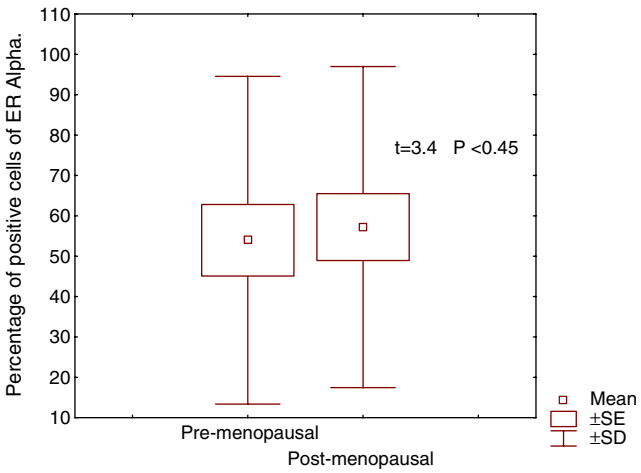

Fig. 2 Cases with positive of ERα according to menopausal status

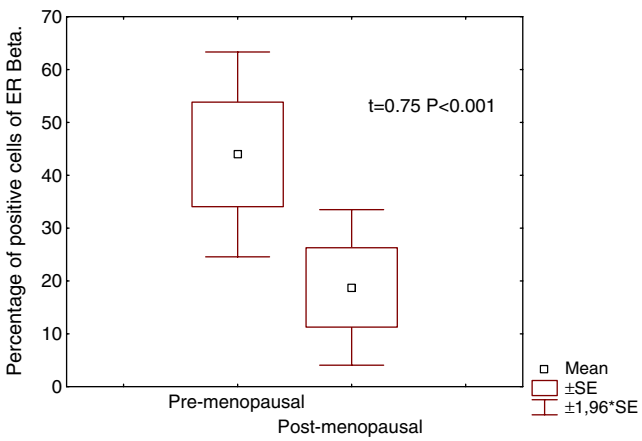

Fig. 3 Cases with positive of ERβ according to menopausal status

Table 4 Hormonal levels and immunohistochemical expression (IHC Score Allred) of estrogen receptors α and β in breast cancer of pre and post menopausal women

Pre-menopausal (n=21)					Post-menopausal (n=23)				
Case	Estradiol	FSH	ERα	ERβ	Case	Estradiol	FSH	ERα	ERβ
1	845.80	0.60	6	7	1	8.60	70.40	7	0
2	319.90	2.00	4	0	2	5.65	63.29	5	0
3	82.10	16.50	8	8	3	5.25	50.17	4	0
4	59.2	73.9	5	8	4	7.80	128.10	8	7
5	72.70	1.60	7	8	5	8.30	69.80	6	0
6	235.80	9.20	0	0	6	22.40	38.40	6	8
7	16.40	44.90	5	8	7	15.40	67.70	4	0
8	245.60	1.60	8	7	8	20.20	49.00	7	6
9	119.00	4.80	6	0	9	13.80	74.30	4	0
10	28.70	6.80	7	0	10	13.90	89.00	8	8
11	928.70	97.10	6	7	11	10.70	52.00	7	0
12	62.00	223.10	8	8	12	13.40	76,.20	8	0
13	44.70	132.30	8	6	13	4.90	11.10	7	0
14	161.70	19.90	7	7	14	27.30	65.30	8	0
15	15.70	82.70	7	0	15	16.50	58.00	7	4
16	35,.00	134.70	7	0	16	25.90	9.40	0	0
17	167.70	44.70	7	6	17	26.40	34.70	7	0
18	92.30	1.00	0	0	18	57.40	29.70	7	7
19	100.10	59.80	4	0	19	15.00	37.80	5	0
20	68.20	4.10	4	0	20	10.60	71.40	0	0
21	31.90	12.30	0	0	21	38.60	55.70	8	0
					22	26.00	101.80	8	0
					23	20.50	38.70	0	0

Correlation Between Hormone Levels and ER-α and ER-β Expression

In pre-menopausal women a correlation was found between serum estradiol level and ER-β. In post-menopausal women a correlation was found between serum FSH level and ER-α (Tables 4 and 5). When serum estradiol was ≥30 pg/ml, ER-α and ER-β showed a positive correlation (r=0.51, p<0.02), but when serum estradiol was <30 pg/ml, this correlation was not observed (r=0.24, p< 0.24).

Table 5 Association of hormonal levels with estrogen receptors

	R	P
ERα		
Estradiol (pg/ml)	0.19	0.89
FSH(UI/L)	0.33	0.02
Estrone (pg/ml)	−0.005	0.97
ERβ		
Estradiol (pg/ml)	0.31	0.03
FSH(UI/L)	0.02	0.88
Estrone (pg/ml)	0.11	0.45

Statistical Power of the Test

The statistical power calculated from our results was 83.13%. We considered the proportional and intensity of ER-β per group. ER-β expression was >80% in 33% of the pre-menopausal women and <50% in 78.2% in the post-menopausal group, in accordance with 0.95 security and a significance level of 0.05. We could conclude that our results were not affected by the sample size.

Discussion

The clinical role of ER-β in breast cancer is controversial in view of recently published results. Inconsistencies among studies are due to heterogeneity in the case series under investigation, such as the number of cases, the clinical condition and the treatment received [8, 12, 13]. The relationship between ER-β and clinico-pathological or biological variables has been addressed in the literature, detecting protein as well as mRNA in case series, including both ER-α-positive and ER-α-negative or only ER-α-positive tumors [14, 15, 17]. ER-β has been associated with favorable outcome prognosis variables: low tumor grade, low S-fraction and negative lymph node status [18–

22]. We found higher ER-β expression in tumor cells from pre-menopausal women than from post-menopausal women. Nevertheless, no differences were found in ER-α expression in either group of women. There was a positive correlation between ER-β and serum estradiol levels in pre-menopausal women. It is important to note that about half the patients were pre-menopausal, a finding in agreement with reports of an increasingly younger onset of disease. Outcome and treatment responses have been related to estrogen receptor status in breast cancer. However, neither the function nor the expression of either molecular form of ER is yet well understood [12]. Recently, differences in estrogen receptors between healthy and tumor breast tissue have been found by PCR and Western blotting [23]. The loss of ER-β expression in tumor tissue has been associated with tumor progression [13, 14, 18, 19]. Moreover, real time PCR was used to investigate the methylation status of the ER-β gene in the ER-β-negative breast cancer cell lines SK-BR-3 and MDA-MB-435; it was found that the ER-β promoter was methylated. The results suggest that loss of ER-β expression is a hallmark of breast carcinogenesis, and that it may be reversible [15]. Zhao et al. [24] also concluded that decreased expression of ER-β mRNA may be associated with breast tumorigenesis and that DNA methylation is an important mechanism for ER-β gene silencing in breast cancer.

However, the specific role in these estrogen receptors is still under investigation [12]. It has been proposed that the balance between ER-α and ER-β is essential for maintaining normal cellular function, and that as ER-β decreases, uncontrolled cellular proliferation leads to metastasis [12]. Our results show that ER-β expression is associated with clinical and biological parameters (age, hormonal status, hormone levels). Some authors [25, 26] have reported that the proportion of ER-α-positive cells in normal breast tissue increases with age, and this observation has been confirmed for ER-β [27]. Meza et al. [11] found that skin-punch biopsies obtained from the external gluteal area of post-menopausal women showed significantly lower levels of ER-α, ER-β and PR than those from pre-menopausal women.

These findings were expected because the sustained diminution of hormonal stimulation brings about a decrease in ER-α and ER-β. In consequence, hormone replacement therapy (HRT) after menopause may coincide with lower expression of ERs in target tissues. These may increase with time [26]. Recently, Hall et al. [28] examined the influence of HRT on the biology of primary breast tumors. Their findings indicate that post-menopausal HRT is associated with distinct expression profiles of 276 genes, related to better recurrence-free survival and lower ER protein levels. Recent studies indicate less aggressive tumor characteristics in HRT users [29–40]. The current study suggests that menopause may influence the expression of both molecular forms of the estrogen receptor in breast cancer. Differences in the expression of ER-β are directly related to the endogenous estrogen level. We found that ER-β was more highly expressed in tumor cells from pre-menopausal women than post-menopausal women. In view of our findings, we could hypothesize that post-menopausal HRT users maintain the profile of ER-β expression in breast tissues, which can be a protective factor. Recently, Nonni et al. [41] evaluated 30 breast tissue specimens from patients whose main lesion was atypical lobular hyperplasia or lobular carcinoma in situ (LN). The Allred score and the percentage of ER-α-positive cells were significantly higher in LN than in the adjacent normal breast tissue. In contrast, the Allred score and the percentage of ER-β-positive cells were significantly lower in LN than in adjacent normal breast tissue. Up-regulation of ER-α and down-regulation of ER-β may represent two discrete molecular events in the pathogenesis of LN [41]. The clinical value of ER-β in cancer prognosis is different for pre- and post-menopausal women. Its possible use for predicting hormonal response should be assessed in large-scale and prospective clinical studies.

Conclusions

These results indicate that estradiol levels in women with breast cancer are associated with ER-β expression in breast tumor tissue; ER-α expression was not associated with hormonal status. The clinical significance of these findings for the prognosis of breast cancer is still to be defined.

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References

1. Deroo BJ, Korach KS (2006) Estrogen receptors and human disease. J Clin Invest 116:561–570
2. Greene G, Gilna P, Waterfield M, Baker A, Hart Y, Shine J (1986) Sequence and expression of human estrogen receptor complementary DNA. Science 231:1150–1154
3. Mosselman S, Polman J, Dijkema R (1996) ERb: Identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49–53
4. Moore J, Mckee D, Slentz-Kesler K, Moore L, Jones S, Horne E et al (1998) Cloning and characterization of human estrogen receptor β isoforms. Biochem Byophis Res Commun; 247:75–78
5. Gustafsson JA (2000) An update on estrogen receptors. Semin Perinatol 24:66–69
6. Hall JL, McDonnell P (1999) The estrogen receptor b isoform (ERb) of the human estrogen receptor modulates ERa transcrip-

tional activity and it a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140:5566–5578
7. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S et al (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors (alpha) and (beta). Endocrinology 138:863–870
8. Speirs V, Skliris GP, Burdall SE, Carder PJ (2002) Distinct expression patterns of ERα and ERβ in normal human mammary gland. J Clin Pathol 55:371–374
9. Murillo-Ortiz B, Perez-Luque E, Malacara JM (2005) Mammographic density and the relationship with the PvuII and XbaI genotypes estrogen receptors alpha (ERa) polymorphisms. Ginecol Obstet Mex 73:229–233
10. Simpson ER, Mahendroot MS, Means GD et al (1994) Aromatase cytocrome P450, the enzyme reponsible for estrogen biosynthesis. Endocrinol Rev 15:342–355
11. Meza-Munoz DE, Fajardo ME, Perez-Luque EL, Malacara JM (2006) Factors associated with estrogen receptors-alpha (ER-alpha) and -beta (ER-beta) and progesterone receptor abundance in obese and non obese pre- and post-menopausal women. Steroids 71(6):498–503
12. Bardin A, Boulle N, Lazennec G, Vignon F, Pujol P (2004) Loss of ERb expression as a common step in estrogen-dependent tumor progression. Endocrine-Related Cancer 11:537–551
13. Lazennec G, Bresson D, Lucas A, Chauveau C, Vignon F (2001) ERb inhibits proliferation and invasion of breast cancer cells. Endocrinology 142:4120–4130
14. Roger P, Sahla ME, Makela S, Gustafsson JA, Baldet P, Rochefort H (2001) Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. Cancer Res 61:2537–2541
15. Qiu C, Shan L, Yu M, Snyderwine EG (2005) Steroid hormone receptor expression and proliferation in rat mammary gland carcinomas induced by 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine. Carcinogenesis 26(4):763–769
16. Harvey JM, Clark GM, Osborne CK, Allred CD (1999) Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 17(5):1474–1481
17. Jarvinen T, Pelto-Huikko M, Holli K, Isola J (2000) Estrogen receptor b is coexpressed with ERa and associated with nodal status, grade, and proliferation rate in breast cancer. Am J Pathol 156:29–35
18. Mann S, Laucirica R, Carlson N, Younes PS, Ali N, Younes A, Li Y, Younes M (2001) Estrogen receptor beta expression in invasive breast cancer. Human Pathol 32:113–118
19. Omoto Y, Inoue S, Ogawa S, Toyama T, Yamashita H, Muramatsu M, Kobayashi S, Iwase H (2001) Clinical value of wild-type estrogen receptor beta expression in breast cancer. Cancer Lett 163:207–212
20. Speirs V, Parkes A, Kerin MJ, Walton DS, Carleton PJ, Fox JN, Atkin SL (1999) Coexpression of estrogen receptor a and b: poor prognostic factors in human breast cancer? Cancer Res 59:525–528
21. Speirs V, Malone C, Walton DS, Kerin MJ, Atkin SL (1999) Increased expression of estrogen receptor b mRNA expression in tamoxifen-resistant breast cancer patients. Cancer Res 59:5421–5424
22. Palmieri C, Cheng GJ, Saji S, Zelada-Hedman M, Warri A, Weihua Z, Van Noorden S, Wahlstrom T, Coombes RC, Warner M, Gustafsson JA (2002) Estrogen receptor beta in breast cancer. Endocr-Relat Cancer 9:1–13
23. Jarzabek K, Koda M, Kozlowski L, Mittre H, Sulkowski S, Kottler M-L, Wolczynski S (2005) Distinct mRNA, protein expression patterns and distribution of oestrogen receptors a and b in human primary breast cancer: correlation with proliferation marker Ki-67 and clinicopathological factors. Eur J Cancer 41:2924–2934
24. Zhao C, Lam WFE, Sunters A, Enmark E, Tamburo De Bella M, Coombes RC, Gustafsson JA, Dahlman-Wright K (2003) Expression of estrogen receptor b isoforms in normal breast epithelial cells and breast cancer: regulation by methylation. Oncogene 22:7600–7606
25. Shoker BS, Jarvis C, Clarke RB, Anderson E, Hewlett J, Davies MP et al (1999) Estrogen receptor-positive proliferating cells in the normal and precancerous breast. Am J Pathol 155:1811–1815
26. Bartow SA (1998) Use of the autopsy to study ontogeny and expression of the estrogen receptor gene in human breast. J Mammary Gland Biol Neoplasia 3:37–48
27. Speirs V, Skliris GP, Burdall SE, Carder PJ (2002) Distinct expression patterns of ER alpha and ER beta in normal human mammary gland. J Clin Pathol 55:371–374
28. Hall P, Ploner A, Björhle J, Huang F, Lin C-Y, Liu ET, Miller LD, Nordgren H, Pawitan Y, Shaw P, Skoog L, Smeds J, Wedrén S, Öhd J, Bergh J (2006) Hormone-replacement therapy influences gene expression profiles and is associated with breast-cancer prognosis: a cohort study. BMC Medicine 4:16
29. Daling JR, Malone KE, Doody DR, Voigt LF, Bernstein L, Marchbanks PA, Coates RJ, Norman SA, Weiss LK, Ursin G, Burkman RT, Deapen D, Folger SG, McDonald JA, Simon MS, Strom BL, Spirtas R (2003) Association of regimens of hormone replacement therapy to prognostic factors among women diagnosed with breast cancer aged 50–64 years. Cancer Epidemiol Biomarkers Prev 12(11 Pt 1):1175–1181
30. Sacchini V, Zurrida S, Andreoni G, Luini A, Galimberti V, Veronesi P, Intra M, Viale G, Veronesi U (2002) Pathologic and biological prognostic factors of breast cancers in short- and long-term hormone replacement therapy users. Ann Surg Oncol 9:266–271
31. Cheek J, Lacy J, Toth-Fejel S, Morris K, Calhoun K, Pommier RF (2002) The impact of hormone replacement therapy on the detection and stage of breast cancer. Arch Surg 137:1015–1021
32. Collaborative Group on Hormonal Factors in Breast Cancer (1997) Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Lancet 350:1047–1059
33. Brinton LA, Hoover R, Fraumeni JF (1986) Menopausal oestrogens and breast cancer risk: an expanded case-control study. Br J Cancer 4(5):825–832
34. Squitieri R, Tartter PI, Ahmed S, Brower ST, Theise ND (1994) Carcinoma of the breast in postmenopausal hormone user and nonuser control groups. J Am Coll Surg 178(2):167–170
35. Magnusson C, Holmberg L, Norden T, Lindgren A, Persson I (1996) Prognostic characteristics in breast cancers after hormone replacement therapy. Breast Cancer Res Treat 38 (3):325–334
36. Bonnier P, Bessenay F, Sasco AJ, Beedassy B, Lejeune C, Romain S, Charpin C, Piana L, Martin PM (1998) Impact of menopausal hormone-replacement therapy on clinical and laboratory characteristics of breast cancer. Int JCancer 79(3):278–282
37. Holli K, Isola J, Cuzick J (1998) Low biologic aggressiveness in breast cancer in women using hormone replacement therapy. J Clin Oncol 16(9):3115–3120
38. O'Connor IF, Shembekar MV, Shousha S (1998) Breast carcinoma developing in patients on hormone replacement therapy: a histological and immunohistological study. J Clin Pathol 51 (12):935–938
39. Carter AC, Sedransk N, Kelley RM, Ansfield FJ, Ravdin RG, Talley RW, Potter NR (1977) Diethylsilbestrol: recommended

dosages for different categories of breast cancer patients Report of the Cooperative Breast Cancer Group. JAMA 237:2079–2085
40. Cappelletti V, Celio L, Bajetta E, Allevi A, Longarini R, Miodini P, Villa R, Fabbri A, Mariani L, Giovanazzi R, Galante E, Greco M, Daidone GM (2004) Prospective evaluation of estrogen receptor-b in predicting response to neoadjuvant antiestrogen therapy in elderly breast cancer patients.. Endocr Relat Cancer 11 (4):761–770, Dec
41. Nonni A, Zagouri F, Sergentanis TN, Lazaris AC, Patsouris ES, Zografos GC (2007) Immunohistochemical expression of estrogen receptors alpha and beta in lobular neoplasia. Virchows Arch 451 (5):893–897, Nov. Epub 2007 Oct 9