Expression of Estrogen Receptors in Relation to Hormone Levels and the Nottingham Prognostic Index

MIA FAHLÉN[1,2,3], HUA ZHANG[3,5], LARS LÖFGREN[1], BRITT MASIRONI[3], EVA VON SCHOULTZ[2], BO VON SCHOULTZ[4] and LENA SAHLIN[3]

[1]Capio St Göran's Hospital, Stockholm, Sweden;
[2]Department of Oncology and Pathology, Karolinska Institutet, Stockholm, Sweden;
[3]Pediatric Endocrinology Unit and [4]Division of Obstetrics and Gynecology, Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden;
[5]Department of Obstetrics and Gynecology, The First Affiliated Hospital of Chongqing Medical University, Chongqing, P.R. China

Abstract. *Background: Estrogen hormones have a large impact on both normal development and tumorigenesis of the breast. Materials and Methods: Breast tissue samples from 49 women undergoing surgery were included. The estrogen receptors (ERα and ERβ), ERα36 and G-coupled estrogen receptor-1 (GPER) were determined in benign and malignant breast tissue. Results: The ERα36 and ERα mRNA levels were highest in malignant tumors. Stromal ERβ immunostaining in benign tumors was higher than in the paired normal tissue. GPER expression was lowest in benign tumors. In the malignant tumors, the Nottingham Prognostic Index (NPI) correlated positively with stromal GPER and the serum testosterone level. The serum insulin-like growth factor-1 (IGF-1) level correlated negatively with GPER mRNA and glandular ERα. Conclusion: The expression of ERα36 is stronger in malignant breast tissue. The strong positive correlation between NPI and GPER in malignant breast stroma indicates an important role for GPER in breast cancer prognosis.*

Estrogen hormones are major contributors to the normal development and maturation of the mammary gland but they are also closely linked to tumorigenesis of the breast (1). Estrogen receptor (ER) signaling is, therefore, a strong target for therapeutics (2). Estrogens typically enhance growth of ER-positive breast cancer but ER interactions with DNA are not necessary for this growth to occur, suggesting that non-genomic actions of ERs may also play a role (3). The two major groups of anti-hormone therapeutics today are tamoxifen, a selective estrogen receptor modulator (SERM) and aromatase inhibitors (AI). SERMs are designed to bind nuclear receptors, whereas the AIs block the production of estrogen, thus diminishing all estrogen action, *i.e.* both *via* receptor-dependent and independent pathways (1).

Estrogen signaling is mediated *via* several receptor proteins. The classical ERα and ERβ are situated in the nucleus, whereas the membrane bound receptor GPER (G-protein coupled estrogen receptor-1; also known as GPR30) and the ERα variant ERα36 are primarily localized to the membrane and cytosol (4, 5). Also, growth factors, *e.g.* IGF-1 (insulin-like growth factor-1) and EGF (epidermal growth factor), have been shown to contribute to the activation of estrogen signaling pathways, acting *via* the so called ligand-independent pathway (4). The IGF-1 system has been shown to regulate GPER expression and function in MCF-7 and Ishikawa cells, triggering the activation of a signaling network that leads to migration and proliferation of cancer cells (6, 7). Estradiol (E2) has been shown to stimulate interaction between GPER and EGF-receptor (7). Thus, a crosstalk between growth factors and ERs is present.

ERα36, a splice variant of ERα, was described in 2005 (8). This new variant differs from the full-length ERα since it lacks both transcriptional activation domains, but still binds to DNA and has part of the ligand-binding domain intact. Additionally, sites of myristoylation are found, suggesting that the receptor is localized in the plasma membrane. The authors predicted that ERα36 could function as a dominant-negative effector of ERα pathways and has a potential to trigger membrane initiated estrogen signaling (8). ERα36 is found expressed in human breast carcinomas and its expression is significantly higher in ER-negative tumors than in ER-positive tumors (8). In addition, ERα36 has been also shown to have an anti-apoptotic effect in triple-negative breast cancer (8).

Correspondence to: Mia Fahlén, Capio St Göran's Hospital, St Göransgatan 1, 113 81 Stockholm, Sweden. Tel: +46 858701000, Fax: +46 858701956, e-mail: mia.fahlen@capiostgoran.se

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Table I. *Serum analyses*.

Median (25th-75th percentile)	All FOLL (n=32)	All LUT (n=17)	Benign all (n=36)	Benign FOLL (n=25)	Benign LUT (n=11)	Malign all (n=13)	Malign FOLL (n=7)	Malign LUT (n=6)
E2 (pmol/l)	402 (128-725)	356 (236-510)	278 (129-522)	206 (118-624)	334 (222-531)	416 (269-559)	537 (300-806)	357 (235-484)
P4 (nmol/l)	3.5 (2.9-5.0)	30.1a (21.6-33.9)	4.6 (3.1-20.1)	3.6 (2.9-4.9)	30.1c (20.3-32.5)	8.7 (3.1-30.6)	3.2 (2.6-6.6)	30.6f (21.2-54.6)
T (total) (nmol/l)	0.90 (0.64-1.11)	0.79 (0.71-1.22)	0.92 (0.72-1.27)	0.93 (0.70-1.32)	0.89 (0.72-1.11)	0.71 (0.53-1.00)	0.58 (0.38-0.98)	0.72 (0.53-1.47)
SHBG (nmol/l)	54.4 (38.5-79.1)	62.5 (41.2-78.8)	54.5 (38.4-79.9)	54.0 (36.8-98.0)	59.9 (40.0-77.4)	67.1 (42.8-73.9)	67.1 (44.0-70.9)	66.5 (41.4-102)
IGF-1 (ng/ml)	148 (118-178)	187b (144-260)	166 (129-244)	150 (124-181)	250d (186-289)	133e (114-166)	127 (83.7-153)	144 (119-182)

a: Significant difference between all FOLL – all LUT; $p<0.001$; b: significant difference between all FOLL – all LUT; $p=0.018$; c: significant difference between benign FOLL– benign LUT; $p<0.001$; d: significant difference between benign FOLL– benign LUT; $p=0.004$; e: significant difference between benign all – malign all; $p=0.025$; f: significant difference between malign FOLL – malign LUT; $p=0.001$; FOLL, follicular; LUT, luteal; E2, estradiol; P4, progesterone; T, testosterone; SHBG, sex hormone-binding globulin; IGF-1, insulin-like growth factor-1.

For many years, the treatment of ERα-positive breast cancer has been dominated by the antagonist tamoxifen (2). In the clinical setting, it has been noted that there seems to occur a form of "tamoxifen resistance" in some breast cancer tumors. With the discovery of additional ERs, like ERα36 and GPER, one explanation might have been found, since tamoxifen is known to act as agonist to ERα36 and GPER in a variety of tumors (5, 7, 8).

The Nottingham Prognostic Index (NPI) is a clinical prognostic guide that has been in use since the late 1970's (9). It is a formula based on nodal status, tumor grade and tumor size. A high NPI score indicates an inferior outcome. It has been proven to be a powerful prognostic tool that has stood the test of time, even comparing to newer types of gene mapping analyses (10).

We hypothesized that there are differences in the presence of the newly described estrogen receptors GPER and ERα36 between normal and tumor breast tissues and that these receptors may have clinical importance. In the present study, the expression of ERα, ERα36, ERβ and GPER is investigated *in vivo* in breast tissue; in benign and malignant tumors and the seemingly normal tissue adjacent to the tumors, *i.e.* paired samples. The results are correlated to serum hormone levels and NPI (9).

Materials and Methods

Patients' characteristics. At the Capio St. Göran's hospital, pre-menopausal women were invited to participate in a study to determine differences between normal, benign and malignant breast tissue regarding expression of receptors in the estrogen-signaling pathway. In addition to collecting paired samples from the same breast (benign/malignant tumor tissue and the seemingly normal tissue adjacent to the tumor), blood samples were obtained from all women to determine hormone levels and the phase of the menstrual cycle (Table I). The inclusion of the women into the study was performed consecutively during a period of 48 months. This resulted in 13 women with primary invasive ductal breast cancer, 29 women with fibroadenoma and 7 women with other forms of benign breast diseases, bringing the total to 49 women. In the group with benign breast disease, there were 25 women in follicular phase and 11 in luteal phase of the menstrual cycle. In the group of women with malignant disease, 7 were in follicular phase and 6 in luteal phase. Data on patients' characteristics, including age, tumor size and the menstrual phase, are given in Table IIa and b.

Breast tissue sampling. All tissue samples were collected in the clinical setting at the time of surgery, when the tumors were removed for therapeutic purpose. For each woman, tissue samples from the tumor and from adjacent seemingly normal tissue, estimated clinically at the operation by the surgeon, were recovered and frozen at –70˚C until analysis. The tissue samples were either extracted for RNA and subsequent polymerase chain reaction (PCR) analyses or embedded in paraffin and cut in 5 μm slices and mounted on glass slides for immunohistochemistry analyses. The tissue slides underwent a re-evaluation by an independent experienced pathologist to confirm the diagnosis of each individual specimen.

RNA preparation and reverse transcription. The frozen breast tissue samples were first transferred to RNAlater-ICE® (Ambion Inc, Austin, TX, USA). Total RNA from the samples was purified with the RNeasy® Lipid Tissue Mini kit (Qiagen GmbH, Hilden, Germany), according to a procedure recommended by the manufacturer for RNA isolation from fatty tissues. Two μg of total RNA from each sample was reverse transcribed at 37˚C for 60 min in a final volume of 30 μl with a reaction mixture (Qiagen) containing 1 x RT buffer, dNTP mix (0.5 mM each dNTP), 600 ng random primers (Invitrogen, Paisley, UK), 2 units RNase inhibitor (Qiagen) and 4 units of Omniscript™ reverse transcriptase (Qiagen).

Real time PCR analysis. Real time PCR was performed in an iCycler™ iQ Real Time PCR System (Bio-Rad Laboratories, Inc.,

Table IIa. *Women with malignant tumors*.

No	Size mm	L/F	Age	S-phase	Histology	Elston	Pos lymph node	NPI	Adj treatment	Paired samples	PCR
1	15	F	41	2%	Ductal	2	N	3.30	Rad, tam	N	Y
2	22	L	40	5%	Ductal	3	Y	5.40	Chemo, rad	Y	N
3	12	F	46	4%	Ductal	2	Y	4.24	Chemo, rad, tam	N	Y
4	10	F	49	2%	Ductal	2	N	3.20	Tam	Y	Y
5	25	L	37	17%	Ductal	2	N	3.50	Chemo, rad, tam	Y	Y
6	10	F	43	0 %	Ductal	2	N	3.20	Chemo, rad, tam	Y	Y
7	20	L	38	5%	Ductal	3	Y	5.40	Declined	Y	Y
8	25	F	46	11%	Ductal	2	Y	4.50	Chemo, rad, tam	N	Y
9	19	F	52	10%	Ductal	3	N	4.40	Chemo, rad, endo	Y	Y
10	14+10	F	42	7%	Ductal	2	N	3.50	Rad, tam	Y	Y
11	25	L	46	1%	Ductal	2	N	3.50	Rad, tam	Y	Y
12	15	L	42	7%	Ductal	3	N	4.30	Chemo, rad, endo	Y	N
13	14+30	L	33	X	Ductal	3	Y	6.90	Chemo, rad endo	Y	Y

X, No information; Y, yes; N, no; chemo, chemotherapy; rad, radiation treatment; tam, tamoxifen; endo, endocrine treatment; F, follicular phase; L, luteal phase; Rec, recurrence; S-phase, proliferation; BC, breast cancer; NPI, Nottingham Prognostic Index; Age, age at operation.

Hercules, CA, USA). For PCR, the cDNAs corresponding to 50–100 ng (see Table III) RNA were added to 12.5 μl of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories) and 0.3 μM of each oligonucleotide primer in a final volume of 25 μl. After initial incubation for 3 min at 95˚C, the samples were subjected to 40 cycles of 10 s at 95˚C, followed by 45 seconds annealing at 55 to 65˚C depending upon the genes (see Table III). All reactions were performed in duplicates. The purity of PCR products was confirmed by a melting curve analysis in all experiments (data not shown). The oligonucleotide primers for *ERα, ERα36, ERβ, GPER* and *RPLP0* (housekeeping gene) are presented in Table III, as well as their predicted sizes. All primers were designed to span an intron/exon boundary or to flank an intron, to eliminate amplification of contaminating DNA. Each PCR assay included a negative control containing a RNA sample without reverse transcription. The primers were based on the sequences of the human genes.

Quantification of mRNA. To standardize the quantification method, *RPLP0* was selected out of several tested housekeeping genes as an invariable internal control (p=0.482). The PCR amplification rate and the cycle threshold (Ct) values were related to a standard curve using iCycler iQ Optical System Software (Bio-Rad). The values of relative expression of genes of interest were normalized against the RPLP0 product.

Immunohistochemistry. The tissue sections were deparaffinized using xylene, rehydrated in graded ethanol and subjected to microwave treatment for antigen retrieval in 0.01 M sodium citrate buffer (pH 6.0) for 10 min and then allowed to cool for a further 20 min at room temperature (RT).

Immunostaining of ERα, ERβ and GPER was performed on 5-μm thick sections using a standard immunohistochemical technique (avidin-biotin-peroxidase) as previously described (11). Before applying the primary antibody, all slides were blocked with normal horse serum diluted in either phosphate-buffered saline or Tris-buffered saline. Thereafter, the primary and secondary antibodies were applied, using dilutions and incubation times as presented in Table IV. After incubation for 30 min with a horseradish peroxidase-avidin biotin complex (Vectastain Elite, Vector, CA, USA), the bound enzyme was visualized by the

Table IIb. *Women with benign tumors*.

No	Size mm	F/L	Age	Histology	Paired samples	PCR
1	23	F	32	FA	Y	Y
2	X	F	31	LCIS*	Y	Y
3	X	F	48	Benign	Y	Y
4	X	F	24	Benign	Y	Y
5	25x30	F	45	Benign	Y	Y
6	12+50	F	36	FA	Y	Y
7	50	L	16	FA	N	Y
8	25	F	31	FA	N	Y
9	X	L	50	FA	Y	Y
10	X	F	32	Sclerosing adenosis*	N	N
11	X	F	24	FA	N	Y
12	X	L	18	FA	N	Y
13	X	F	42	FA	Y	Y
14	X	F	21	FA	N	N
15	X	F	36	FA	Y	Y
16	X	F	31	FA	Y	Y
17	X	F	50	FA	N	N
18	X	F	48	FA	N	N
19	X	L	22	FA	Y	Y
20	X	L	37	FA	Y	Y
21	X	L	41	FA	Y	Y
22	X	L	32	FA	N	Y
23	X	F	37	FA	N	Y
24	X	L	38	FA	Y	Y
25	X	F	20	FA	N	N
26	X	F	26	FA	Y	Y
27	X	F	16	FA	Y	Y
28	X	L	29	FA	Y	Y
29	X	L	51	FA	Y	Y
30	X	F	33	FA	Y	Y
31	X	F	20	FA	N	N
32	X	F	43	FA	Y	Y
33	X	F	26	FA	Y	Y
34	X	L	40	FA	Y	Y
35	X	F	48	Benign	Y	Y
36	X	F	23	FA	Y	Y

X, No information; Y, yes; N, no; FA, fibroadenoma; F, follicular phase; L, luteal phase; Age, age at operation; LCIS, Lobular cancer *in situ*; *LCIS and sclerosing adenosis are considered benign in clinical practice.

Table III. *Oligonucleotide primers used for real-time PCR, template amount and their annealing temperatures.*

Gene	Accession No. or Reference	Primers F=forward; R=reverse	Position	cDNA	Annealing step
ERα ESR1	NM_000125	F: CTTGCTCTTGGACAGGAACC R: TCCTCTCCCTGCAGATTCAT	bp 1455-1474 bp 1565-1546 product: 111 bp	50 ng	57˚C/45 s
ERα-36	BX640939.1	F: TCCTCGTGTCTAAAGCCTCTGGT R: GCCCATGGTCATGTAACTGCCT	bp 1154-1176 bp 1264-1243 product: 111bp	50 ng	57˚C/45 s
ERβ ESR2	NM_001437.2	F: GGCATGCGAGTAACAAGGGCA R: GAAGCACGTGGGCATTCAGCA	bp 1865-1885 bp 1969-1949 product: 105 bp	100 ng	55˚C/45 s
GPER	NM_001505.2	F: GTGGGGAAGAGGCCACCAACATCTG R: ACTCTCTGGGTACCTGGGTTGCAGC	bp 406-430 bp 575-551 product: 170 bp	100 ng	65˚C/45 s
RPLP0	NM_001002.3	F: GGCGACCTGGAAGTCCAACT R: CCATCAGCACCACAGCCTTC	bp 195-214 bp 343-324 product: 149 bp	50 ng	62˚C/45 s

Table IV. *Antibodies used in the study, their concentrations, buffers and incubation times.*

Protein	Blocking RT	Primary antibody	Primary antibody type	Primary antibody dilution	Incubation	Secondary antibody (diluted 1:200) RT	Buffer for secondary antibody dilution
ERα	1.5% NHS 30 min	Zymed 08 -1149	Monoclonal mouse IgG	1:5	4˚C overnight	Biotinylated Horse anti Mouse IgG[1] 30 min	1.5% NHS in PBS
ERβ	5% NHS + 5% BSA 45 min	Serotec MCA1974	Monoclonal mouse IgG	1:20	4˚C overnight	Biotinylated Horse anti Mouse IgG[1] 45 min	5% NHS in TBS+ 5% BSA
GPER	1.5% NHS 30 min	Atlas HPA027052	Polyclonal Rabbit IgG	1:250	4˚C overnight	Biotinylated Horse anti Mouse/Rabbit IgG[2] 30 min	1.5% NHS in PBS

NHS, Normal horse serum; BSA, bovine serum albumin; RT, room temperature; [1]Vector laboratories, catalog no. BA-2000; [2]Vector laboratories, catalog no. BA-1400

application of 3,3'-diaminobenzidine (DAKO Cytomation, Carpinteria, CA, USA). The sections were counterstained with hematoxylin and dehydrated before mounted with Pertex®. We have not found any commercial antibody that works for immunohistological analysis of ERα36.

Image analysis. To assess the ERα immunostaining quantitatively, a Leica microscope was connected to a computer using a Colorvision software (Leica Imaging system Ltd. Cambridge UK). Ten fields were randomly selected from the glandular tissue in a systematic way for quantification of the area of positively immunostained nuclei. The stromal tissue was actively excluded from the measurements. In a few samples, there was not possible to obtain measurements from ten separate sites due to lack of tissue. In those few cases, all glandular tissue was measured. Using color discrimination software, the total area of positively stained nuclei (brown reaction product) was measured and expressed as a ratio of the total area of cell nuclei (brown reaction product + blue hematoxylin).

Manual scoring. ERβ and GPER immunostainings were assessed by manual scoring due to their character of staining, *i.e.* not only nuclear. The scoring was performed by two independent observers on a four-point scale from negative (0), (+) faint, (++) moderate to (+++) strong immunostaining. The results by this method, from two independent observers, show a good consistency between investigators (11).

Statistical analysis. Statistical analysis was performed by ANOVA on ranks (Kruskal-Wallis test) and significances were evaluated by the Dunn's test. When two samples were compared, the Mann Whitney Rank Sum Test was used. Paired analyses were run by the Wilcoxon's Signed rank test. Correlations were evaluated by the Spearman's test. Values were considered significantly different when $p<0.05$.

Hormone analyses. The hormone levels were established using venous blood samples obtained at the time of surgery. Serum concentrations of sex hormone-binding globulin (SHBG) and E2 were determined by chemiluminiscence enzyme immunoassay

using commercial kits obtained from Siemens (Immulite® SHBG and Estradiol; Diagnostic Products Corp., Los Angeles, CA, USA). The serum concentration of IGF-1 was determined by an enzyme-labeled chemiluminescent immunocentric assay using a commercial kit obtained from Siemens (Immulite® IGF-1; Diagnostic Products Corp.). Progesterone (P) was determined by sequential competitive immunoassay, using a commercial kit obtained from Siemens (Immulite® Progesterone Diagnostic Products Corp.). Total levels of testosterone were determined by a radioimmunoassay (RIA) using a commercial kit obtained from Orion (Spectria® Testosterone RIA; Orion Diagnostica OY, Esbo, Finland). All analyses were performed according the manuals from the manufacturers. The concentration of free testosterone (fT) was calculated from the value of total testosterone, SHBG and a fixed albumin concentration of 40 g/l by successive approximation using a computer program based upon an equation system derived from the law of mass action (12). Detection limits and coefficients of variation for within and between assays were: 0.1 U/l, 5.7% and 12.3% for luteinizing hormone (LH); 0.1 U/l, 3.0% and 5.9% for follicle-stimulating hormone (FSH); 0.2 nmol/l, 6.5% and 8.7% for SHBG; 55 pmol/l, 9.3% and 10.6% for E2; 20 ng/ml, 3.6% and 6.6% for IGF-1; 0.11 nmol/l, 5.3% and 5.4% for testosterone; 0.6 nmol/l, 9.6% and 9.9% for progesterone, respectively.

Ethics and consent. The study was approved by the Local Ethics Committee at Karolinska Institutet, Stockholm, Sweden (98-173). All women gave their written consent to participate in the study.

Results

Hormone analyses. Beside the expected result of higher progesterone levels in serum from women in luteal phase as compared to follicular phase, we observed a higher median IGF-1 level in the women in luteal phase compared with those in follicular phase (187 *vs*. 148 ng/ml; p=0.018; Table I). When comparing all women with benign tumors to those with malignant, we found a higher level of IGF-1 in those with benign tumors (166 *vs*. 133 ng/ml; p=0.025; Table I).

Positive correlation was found between estradiol and testosterone (r=0.424; p=0.013) in blood samples (n=34) from all women, while negative correlation was found between SHBG and testosterone (r=-0.342; p=0.048).

The *ERα* mRNA level was higher in malignant (M) tumors as compared to benign (B) tumors and normal (N) tissue (p=0.001; n=71) (Figure 1, top panel).

ERα immunostaining in glandular epithelium (GE) was higher in M tumors as compared to B tumors (p=0.007; n=83) (Figure 2A-C; Figure 3, top panel). In paired analysis of B tumors we found that the ERα protein in GE was less expressed in the tumor as compared to N tissue (48% *vs*. 64%; p=0.009; n=23). In paired analysis of M tumors, we found no significant differences.

The *ERα36* mRNA levels were higher in M tumors (n=11) as compared to normal tissue (n=27) (p=0.002) (Figure 1; next to top panel). The benign tissue (n=29) did not differ to any of the other groups. In paired comparison, M tumors expressed more *ERα36* mRNA as compared to their matched seemingly normal tissue (n=8; p=0.008).

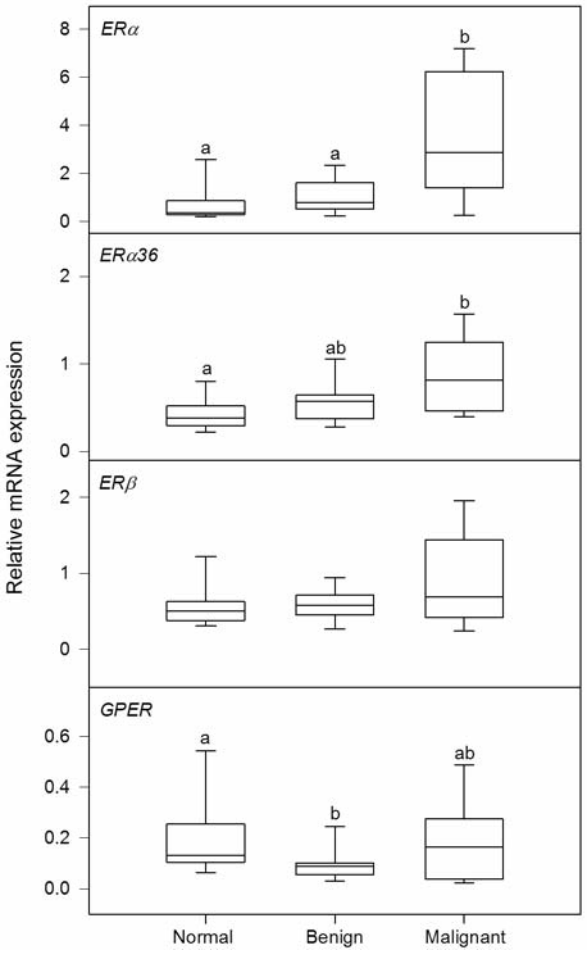

Figure 1. *mRNA determinations by real time PCR, for ERα (top panel), ERα36 (upper middle panel), ERβ (lower middle panel) and GPER (bottom panel). The groups are the seemingly normal tissue adjacent to tumors, as well as the benign and malignant tumors. The n values are for the normal tissue=30; benign tumors=30 and malignant tumors=10. The box-plots show the median with 50% of the data falling within the box. The whiskers extend to the 5th and 95th percentiles. Boxes with different letter designations are significantly different, i.e. p<0.05.*

The *ERβ* mRNA levels did not differ between groups (p=0.177; n=71) (Figure 1; next to bottom panel).

ERβ immunostaining (Figure 2 D-F) was manually scored and GE, stroma and vessels were separately assessed. There were no differences in ERβ immunostaining between groups for any of the cell types. In paired analyses of B tumors, the stromal ERβ protein was increased in the tumor (p=0.027; n=22 pairs).

The *GPER* mRNA level was lower in B tumors than in N tissue (p=0.003; n=71) (Figure 1, bottom panel). We found *GPER* mRNA expression to be less in B tumors than in the

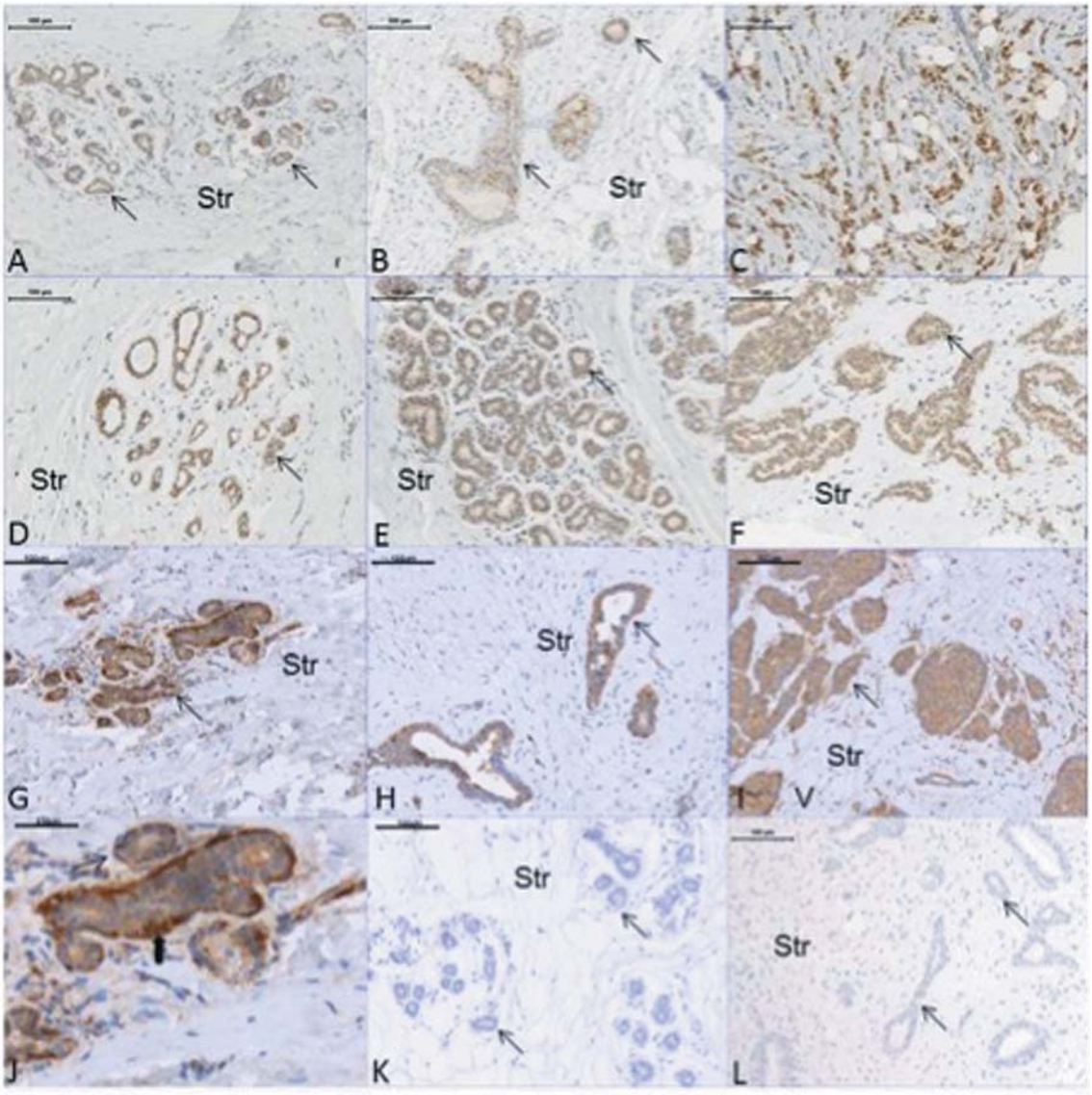

Figure 2. *Immunohistochemical images from normal (left column), benign (middle column) and malignant (right column) breast biopsies. The tissues were stained for ERα (A-C), ERβ (D-F) and GPER (G-J). The negative controls (K-L), where the primary GPER polyclonal antibody was replaced by a similar concentration of rabbit IgG (K, normal tissue) and the monoclonal antibody (ERα/ERβ) was replaced by mouse IgG at an equivalent concentration (L, benign tumor), are also shown. Str=Stroma, thin arrows point at glandular epithelium and a thick arrow points at myoepitelial cells in (J). All figures, but J, are shown at magnification ×200, scale bar=100 μm. J has magnification ×500, scale bar 250 μm.*

paired N tissue (p=0.032; n=22 pairs). There were no differences in paired analyses of M tumors.

GPER immunostaining (Figure 2G-J) was manually scored and results were obtained for GE, stroma, vessel and myoepithelial (ME) cells around the glands. There was a tendency of lower GPER immunostaining in the GE from B tumors compared to M and N samples (p=0.056; n=83). There were no differences found in the stroma; however, in vessels, B tumors showed less immunostaining as compared to N tissue (p=0.001; n=83) (Figure 3; middle panel). GPER immunostaining of the ME cells was different between groups (p<0.001; n=63) and compared to the N group the B tumors showed less immunostaining (Figure 3; bottom panel). No differences were found in paired analyses of B tumors in GE and stroma. The GPER protein was higher in N tissue as compared to B tumors both in vessels (p=0.007; n=22) and in ME cells (p=0.008; n=17).

In benign tumors there is a positive correlation between the serum IGF-1 level and GPER immunostaining in myoepithelial cells (r=0.449; p=0.031; n=23).

In malignant tumors we found a negative correlation between levels of serum IGF-1 and *GPER* mRNA (r=–0.804; p=0.001; n=11) but a positive correlation between NPI and stromal GPER immunostaining (r=0.814; p=0.004; n=9), as well as NPI and the serum testosterone level (r=0.682; p=0.019; n=11).

Discussion

This study aims to clarify the similarities and differences between breast tissues in variant forms of differentiation, in particular in relation to the membrane bound estrogen receptors ERα36 and GPER. We divided the samples in three categories, malignant, benign and normal. They were compared as total groups but also in paired samples where the women were their own controls.

The correlation found between total testosterone, estradiol and SHBG is in good agreement with Pasquali *et al*. who showed SHBG to be negatively correlated with testosterone levels in pre- and postmenopausal women (13). The higher level of IGF-1 in the luteal phase as compared to follicular is also concurrent with a previous report (14).

Our data show the highest levels of *ERα* mRNA in malignant tumors, which is in agreement with previous data on postmenopausal women (15). The protein expression of ERα in glandular cells was also higher in malignant tissue as compared to benign but was not different to normal tissue. ERα is a receptor that has been known for many years as an important factor for growth and maturation of the normal breast and its involvement in cancer development has also been acknowledged since the early sixties (2). ERα has been shown to be related to tumor growth and, also, the main therapeutic target for SERMs.

Concerning ERα36, a receptor that has been described to be involved in tamoxifen resistance (5, 8), our data showed a higher *ERα36* mRNA level in malignant tumors as compared to normal tissue. Furthermore, the *ERα36* mRNA level was higher in malignant tumors also in paired analysis. This receptor was first identified as a splice variant of ERα (5, 8). It is reported to affect cancer cell survivability, promote anti-apoptosis and reduce the effect of taxol (8). Some authors argue that ERα36 does not exist in normal mammary breast epithelium (16) but, although lowest in normal tissue, we could still detect *ERα36* mRNA in the present study. This might be due to the fact that our seemingly normal tissue is obtained from a breast with a tumor disease and, therefore, not completely normal.

We found no significant differences in the expression of *ERβ* mRNA in the present study; however, in paired analysis of benign tumors, stromal ERβ immunostaining was higher in the tumors than in the normal tissue. ERβ has added some conflicting data to the knowledge of breast cancer. The role of ERβ in normal tissue (17), in malignant tissue and its part in tamoxifen resistance remain utterly conflicting (18, 19). Most studies claim that ERβ is a marker of good prognosis. It is present in the majority of normal breast cells and its presence diminishes whilst the breast cancer progresses. It seems to have a positive effect on apoptosis and chemotherapy treatment, as well as to suppress cell proliferation; however, other studies present opposite opinions (20).

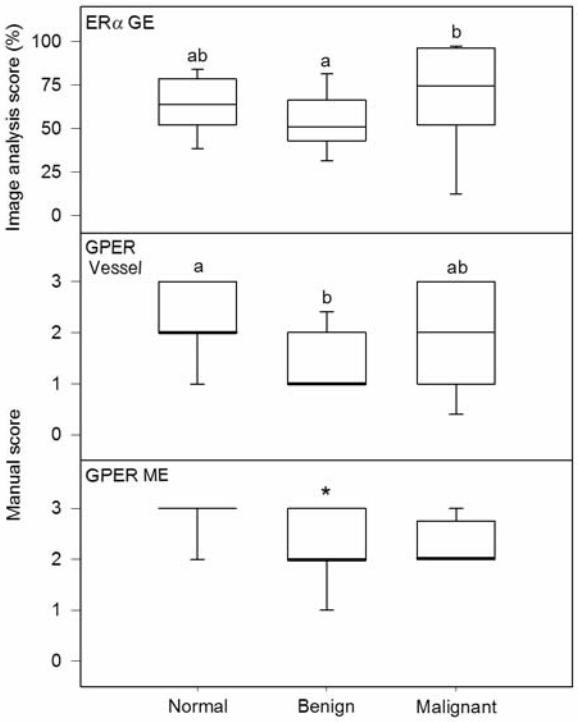

Figure 3. *Results as assessed by image analysis and manual scoring from immunohistochemistry images. From top to bottom: ERα glandular epithelium (GE); GPER vessel and GPER myoepithelial cells (ME). The n values are for Normal=35; Benign tumors=35 and Malignant tumors=13 in all assays, but the ME, where Normal=32, Benign=27 and Malignant=4 since the cells were not present in some of the samples and not able to be determined in most of the malignancies. The box-plots show the median with 50% of the data falling within the box. The whiskers extend to the 5th and 95th percentiles. Boxes with different letter designations are significantly different. The asterisk shows the significant difference of ME cells from benign tumors as compared to the normal tissue.*

We found that *GPER* mRNA was decreased in benign tumors compared to normal tissue for all samples, also when paired. The GPER protein showed a similar tendency. GPER is a membrane-associated receptor implicated in a variety of actions *e.g.* potentiation of cancer growth *via* the induction of the connective tissue growth factor (CTGF), which in turn promotes migration. Studies show that GPER may be induced by IGF-1 and that it is involved in proliferation and promoting invasion of inflammatory breast cancer (6, 7, 21). This is in

agreement with our finding of GPER immunostaining in myoepithelial cells, with IGF-1 being positively correlated in benign tumors. On the other hand, we found *GPER* mRNA expression negatively correlated to IGF-1 in malignant tumors. The reason for this discrepancy could be that the situation *in vivo*, in our present study, is not similar to the *in vitro* situation in the studies on MCF-7 and Ishikawa cancer cells or in IBC cell lines SUM149 and SUM190 (6, 21).

The normal myoepithelial cells are assumed to act as a barrier, being a part of the basal layer and expressing tumor suppressor proteins, *e.g.* p63 and p73 (22). It has also been shown that tumor-derived myoepithelial cells differ from those in the normal tissue and loose some of these abilities. One study showed that myoepithelial cells might play a role in the switch from *in situ* cancer to invasive cancer (23). This concurs with our findings that the GPER protein was higher in normal tissue as compared to benign tumors in vessels and, in particular, the ME cells. Thus, there is a striking difference in GPER expression between benign and malignant tumors and it is likely that GPER expression in the myoepithelial cells could have impact on metastatic potential. GPER has, just as ERα36 (see above), been suggested as part of the tamoxifen resistance process (7), which is another possible link to the association with NPI. Our finding that NPI is being positively correlated to stromal GPER indicates that this receptor might be important for the clinical outcome. Indeed, a study on *Gper* knockout (KO) mice shows that GPER has a critical role in breast tumor growth and metastasis. The authors described that the tumors in the *Gper* KO mice were smaller, had decreased proliferation, lower histological grade and fewer lung metastases compared with wild type mice (24).

The strength of the present study is the paired sampling, the women being their own controls, and that these premenopausal women were not hormone-treated at the time of surgery. The weakness is the relatively small number of patients.

The novel insights into the role of Gper expression in breast cancer growth in mice (24) together with the data from this study in humans, definitely stress the importance of GPER in breast cancer development and prognosis. In addition, ERα36 and GPER are both involved in tamoxifen resistance, albeit in different ways (5, 7, 8). When GPER is expressed in the stroma, another anti-estrogen therapy might be preferable, *e.g.* aromatase inhibitors. The expanded insight into how these different aspects of estrogen receptors work in normal, benign and malignant tissues of the human breast opens up for a variety of new targeted treatments, such as monoclonal antibodies. It might also give us more powerful prognostic tools to better tailor the treatment for the vast number of women with breast cancer.

Conflicts of Interest

None to declare.

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