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# Tissue estradiol is selectively elevated in receptor positive breast cancers while tumour estrone is reduced independent of receptor status

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ABSTRACT

Previous studies have suggested elevated estrogen production in tumour-bearing breast quadrants as well as in breast cancers versus benign tissue. Using highly sensitive assays, we determined breast cancer tissue estrogen concentrations together with plasma and benign tissue estrogen concentrations in each quadrant obtained from mastectomy specimens (34 postmenopausal and 13 premenopausal women). We detected similar concentrations of each of the three major estrogens estradiol ($E_2$), estrone ($E_1$) and $E_1S$ in tumour-bearing versus non-tumour-bearing quadrants. Considering malignant tumours, intratumour $E_1$ levels were reduced in cancer tissue obtained from pre- as well as postmenopausal women independent of tumour ER status (average ratio $E_1$ cancer: benign tissue of 0.2 and 0.3, respectively; $p < 0.001$ for both groups), suggesting intratumour aromatization to be of minor importance. The most striking finding was a significant (4.1–8.6-fold) increased $E_2$ concentration in ER positive tumours versus normal tissue ($p < 0.05$ and $<0.001$ for pre- and postmenopausal patients, respectively), contrasting low $E_2$ concentrations in ER− tumours ($p < 0.01$ and $<0.001$ comparing $E_2$ levels between ER+ and ER− tumours in pre- and postmenopausals, respectively). A possible explanation to our finding is increased ligand receptor binding capacity for $E_2$ in receptor positive tumours but alternative factors influencing intratumour estrogen disposition cannot be excluded.

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## 1. Introduction

Estrogen levels play a pivotal role to breast cancer development as well as therapy. Recent studies have linked postmenopausal plasma estrogen levels to subsequent risk of breast cancer development [1], and estrogen deprivation with use of aromatase inhibitors is currently replacing treatment with the antiestrogen tamoxifen as adjuvant therapy in postmenopausal patients [2].

The finding that successful treatment with aromatase inhibitors is correlated to the degree of aromatase inhibition [3] underlines the importance of studying estrogen homeostasis in postmenopausal women in general and in breast cancer patients in particular. The pathway of estrogen synthesis involving aromatization of androgens into estrogens is well characterized [4]; in contrast, endogenous mechanisms controlling estrogen synthesis are poorly understood. While several investigators have determined plasma estrogen levels in postmenopausal breast cancer patients, due to methodological difficulties the number of studies on tissue estrogen levels is limited, and the number of patients enrolled is low [5–14], producing conflicting results. It has been known for two decades that intratumour estradiol ($E_2$) levels may exceed plasma levels, in particular in postmenopausal women [6]. Thus, increased local production [13] as well as enhanced estrogen uptake from the circulation [15] has been proposed to explain this phenomenon.

Miller and colleagues [12] two decades ago found higher aromatase activity in normal tissue obtained from the tumour-bearing breast quadrant when compared to the other three quadrants of the same breast, suggesting local estrogen production in benign breast tissue may enhance tumour growth. Recently, we developed a highly sensitive assay [16] allowing us to determine tissue levels of the three major estrogen fractions; estradiol ($E_2$), estrone ($E_1$) and estrone sulphate ($E_1S$) simultaneously. Notably, tissue as well as plasma estrogen levels are subject not only to aromatization; balance between $E_2$, $E_1$ and its sulphate conjugate $E_1S$ is regulated by complex enzyme systems including different hydroxysteroid dehydrogenases facilitating steroid reduction versus oxidation [17–21] as well as enzymes regulating sulphate conjugation [22]. An

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improved understanding of the relationship between intratumour estrogen levels and intrinsic tumour biological factors on the one hand versus benign tissue and plasma estrogen concentrations on the other may provide important background information to further studies in this area.

In this study, we explored several issues with respect to estrogen disposition in breast cancer. To evaluate potential contribution of local breast synthesis to breast cancer growth, we determined tissue estrogen levels in the tumour-bearing breast quadrant and compared hormone levels to those obtained in the non-tumour-bearing quadrants of the same breast.

Second, to address potential contribution from benign breast synthesis versus circulating estrogens to intratumoural hormone levels, we measured tumour estrogen levels and correlated individual parameters to benign tissue and plasma hormone levels. Breast density is a strong risk factor to subsequent breast cancer development [23] probably exceeding plasma estrogen levels. Aromatase expression is regulated by different promoters in benign breast compared to other body compartments [24]; thus, an intrinsic propensity for high local breast estrogen synthesis may be a risk factor for breast cancer development separate from circulating hormone levels.

Third, we related tumour estrogen levels to estrogen receptor status in the tumours.

Finally, we questioned whether plasma and/or benign tissue estrogen levels may be higher among patients harbouring ER+ compared to ER− tumours. If so, this may be consistent with the hypothesis that plasma estrogens promote hormone-sensitive tumour development in a concentration-related manner.

While we detected elevated $E_2$ levels in ER+ tumours in pre- as well as in postmenopausal women, surprisingly intratumour $E_1$ was consistently lower compared to benign tissue levels independent of ER and patient menopausal status. These findings challenge the importance of intratumour aromatization, suggesting other mechanism explaining elevated $E_2$ levels in ER+ tumours.

## 2. Patients, materials and methods

### 2.1. Patients

Pre- and postmenopausal breast cancer patients selected for mastectomy at the Department of Surgery (Haukeland University Hospital, Bergen, Norway) were asked to participate in this study. Exclusion criteria were any kind of hormone replacement therapy that was not terminated for at least 4 weeks prior to inclusion as well as any drug known to interfere with estrogen disposition. Four patients were found to have taken hormone replacement therapy within the 4 weeks pre-surgical interval. The values from these patients are depicted (and marked with an asterisk) as individual values in Fig. 3a–c; except from that, they were excluded from all data analysis. The patients were informed about the intention of the study, and gave their written informed consent prior to inclusion according to National Regulations. Demographic data (age, height and body weight; BW) were collected, after which the database was anonymised.

### 2.2. Tissue and blood specimen sampling

Tissue (benign and breast cancer) samples for estrogen measurements were obtained from mastectomy specimens in the theatre immediately upon removal and snap-frozen in liquid nitrogen. About 500 mg of tumour tissue and an equivalent amount of benign tissue was obtained from each of the four breast quadrants. All tissue samples were subsequently stored in liquid nitrogen until processing.

Blood samples for plasma hormone measurements were drawn on the morning of the day of surgery following an overnight fast. Briefly, 20 ml blood was collected in heparinised vials (2 vials containing 10 ml each). Plasma was separated by centrifugation and stored at −20 °C until processing.

### 2.3. Reagents

Plasma levels of estradiol ($E_2$), estrone ($E_1$), and estrone sulphate ($E_1S$) were determined using a highly sensitive radioimmunoassay recently updated with respect to sensitivity [25]. Radiolabelled estrogens for recovery measurements [2,4,6,7-$^3$H]$E_1$ (101 and 100 Cl/mmol) and [2,4,6,7,16,17-$^3$H]$E_2$ (154 and 137 Ci/mmol) were obtained from Amersham Biosciences, UK (Little Chalfont, Buckinghamshire), while [6,7-$^3$H]$E_1S$ (50 and 53 Ci/mmol) was obtained from American Radiolabelled Chemicals Inc. (St. Louis, MO), Estradiol-6-(O-carboxymethyl)-oximino-2-[$^{125}$I]-iodo-histamine (2000 Ci/mmol) was provided from Amersham Biosciences, UK (Little Chalfont, Buckinghamshire). Estrone 3-sulfate sodium salt (E0251) was provided by Sigma Chemicals Co. (London, UK). Sephadex LH-20 was obtained from Amersham Biosciences AB (Uppsala, Sweden), sulfatase (S-9754) from Sigma, and the $E_2$ antibody (ER 150), Sorin Biomedica S.p.A., Saluggia, Italy from Sodiag SA (Losone, Switzerland). Methanol, dichloromethane (DCM) and ethyl acetate were obtained from Merck AG (Darmstadt, Germany). All these reagents were of HPLC grade. Ethanol was obtained from Arcus A/S (Oslo, Norway). Sodium borohydride was from Fluka Chemie AG (Buchs, Switzerland) and disodiumhydrogenphosphatedihydrate as well as sodium azide were from Merck AG.

### 2.4. Plasma hormone measurements

To determine plasma estrogen levels, about 2000 dpm of [$^3$H]$E_2$, [$^3$H]$E_1$, and [$^3$H]$E_1S$ for internal recovery standards was added to the test tubes and the plasma sample (2 ml) added. The resulting suspension was incubated at 4 °C overnight. Unconjugated estrogens were extracted with ether followed by chromatography on LH-20 columns. The fraction containing $E_1$ and $E_2$ was eluted on a LH-20 column using dichloromethane:ethyl acetate:methanol (97:5:1 by vol.) as solvent. The $E_2$ aliquots were evaporated to dryness in the test tubes followed by RIA. This was performed using iodinated tracer at a concentration of about 17,000 cpm per sample as previously reported [25]. Because commercially available RIAs using a $^{125}$I isotope has not been validated for $E_1$ measurements in the very low range, and RIAs using [$^3$H]$E_1$ lack the sensitivity required [26], plasma $E_1$ fractions were converted in to $E_2$ using a method described previously [27] and determined similar to $E_2$ as previously outlined [25].

To determine the concentration of plasma $E_1S$, following the extraction of unconjugated estrogens, ethanol (5 + 3 ml) was added to the water fractions. The extracts were pooled, evaporated to dryness, and the residue reconstituted in 2 ml sodium acetate buffer (0.2 M, pH 5) containing sulphatase to a final concentration of 0.2 mg/ml. Following incubation at −37 °C for 24 h, samples were extracted, and the $E_1$ fraction handled as outlined above (see [25] for details).

Details with respect to recovery as well as CV of this method and detection limits are reported elsewhere [25]. The overall recovery through all purification steps was on mean 88%, 90% and 49% for $E_1$, $E_2$ and $E_1S$, respectively, with corresponding coefficients of variation <5% for $E_1$ and $E_2$ in the low- as well as high-concentration range, and 5.1–6.2% with respect to $E_1S$. The amount of [$^3$H]$E_1$, [$^3$H]$E_2$ and [$^3$H]$E_1S$ added to the samples contributed about 4.50, 2.95 and 9.09 pmol/L to the results. Thus, the final results have been corrected for these contributions caused by the recovery proce-

**Table 1**
Key demographic data for the patient population studied.

| | Postmenopausal patients | | Premenopausal patients |
|---|---|---|---|
| | −HRT[a] $n = 30$[b] | +HRT[a] $n = 4$ | $n = 13$[c] |
| Age at surgery: median (min, max) | 58 (44–81) | 52 (52–54) | 39 (31–49) |
| BMI: Geom. mean w.95% Conf. int. | 24.6 (22.8–26.5) | 22.0 (13.5–36.0) | 23.2 (20.4–26.3) |

[a] HRT +/− receiving hormone replacement therapy or no hormone replacement within last 4 weeks.
[b] 2 central located tumours.
[c] 3 central located tumours.

dures. Further, the method was validated by adding unlabelled $E_1$, $E_2$ and $E_1S$ in different concentrations [25]. Intra-assay CV for estrogen levels in the plasma concentration range for postmenopausal women were <5% for $E_2$ and <10% for $E_1$ and $E_1S$, and detection limits were 0.67, 1.14 and 0.55 pmol/L for $E_2$, $E_1$ and $E_1S$, respectively.

Plasma levels of androstenedione (A) were measured using the commercial kit provided by Diagnostic Systems Laboratories Inc., Webster, TX, USA (DSL-3800 ACTIVE Androstenedione Coated-Tube Radioimmunoassay). The detection limit for A was 0.03 ng/ml. Plasma testosterone (T) was measured using a commercial coated tube RIA-kit provided by Diagnostic Systems Laboratories, Webster, TX (DSL-4000). The theoretical sensitivity limit according to the manufacturer is reported as 0.08 ng/mL.

### 2.5. Tissue hormone measurements

Breast tissue estrogen levels were determined using a highly sensitive HPLC-RIA developed in our laboratory outlined with a detailed flow sheet elsewhere [16]. Briefly, tumour tissue samples were homogenized using an Ultra Turrax T25 and incubated with $[^3H]E_2$, $[^3H]E_1$, and $[^3H]E_1S$ (about 2000 dpm each) for internal recovery calculations. The suspension was incubated for 24 h at 4 °C. To enhance equilibrium, samples were treated with ultrasound before extraction of the unconjugated estrogens with ether and lipidex 5000 column chromatography. The individual unconjugated estrogen fractions were subsequently separated by high pressure liquid chromatography. Similar to what was done with plasma samples, the $E_1$ fraction was converted into $E_2$ using sodium borohydride, ether extracted and purified on a LH-20 column. To determine the concentration of tissue $E_1S$, ethanol was added to the water fraction following the extraction of the free estrogens. The ethanol fraction was removed, dried, and the residue reconstituted in 2 ml sodium acetate buffer (0.2 M, pH 5) containing sulphatase to a final concentration of 2.0 mg/mL. Following incubation at 37 °C for 48 h, free $E_1$ was removed by ether extraction and purified by LH-20 column chromatography. Thereafter, $E_1$ was converted into $E_2$, ether extracted, purified by high pressure liquid chromatography, ether extracted, and purified on a LH-20 column prior to measurement by the $E_2$-RIA. For the $E_2$-RIA, the $E_2$-fractions ($E_2$, as well as $E_2$ converted from $E_1$ or $E_1S$) were re-constituted in 1 ml methanol. 300 μl was obtained for recovery measurements, and aliquots of 300 μl used in duplicate for the RIA [16]. The detection limits are 4.3 fmol/g tissue for $E_2$, 19.8 fmol/g tissue for $E_1$ and 11.9 fmol/g tissue for $E_1S$. The all-over recovery of the individual steroids through the purification steps were 25–50% for $[^3H]E_1$ and $[^3H]E_2$ and 15–30% for $[^3H]E_1S$. Final values were corrected for individual recovery and the amount of $[^3H]$-labelled hormone added as individual standard. Repeated analysis on several ($n = 4$) occasions of multiple samples (8–10) obtained from an individual tumour revealed an intra-assay CV of 6.0%, 6.7% and 6.4% for $E_2$, $E_1$ and $E_1S$, respectively, with an inter-assay CV of 19.0%, 16.0% and 20.1%, respectively [16].

### 2.6. Statistical methods

Plasma and tissue levels of $E_2$, $E_1$ and $E_1S$ are reported as their geometrical mean value with 95% confidence interval (CI). $E_2$, $E_1$ and $E_1S$ in the tissue from the four quadrants were initially compared using the Friedman test. Thereafter, the value from the tumour-bearing quadrant was compared to the mean level of the three other quadrants using the Wilcoxon-Matched Pair Signed rank test. Statistical differences in hormone or hormone ratio levels between ER+ and ER− tumours were evaluated with use of the Mann–Whitney test. Correlations between individual levels of plasma, benign and malignant tissue hormone levels were tested for using the Spearman rank test.

## 3. Results

A total of 47 breast cancer patients (34 postmenopausal and 13 premenopausal) were enrolled. Among these, 4 postmenopausal patients had received hormone replacement therapy within the last 4 weeks prior to enrolment. These patients were excluded from the general statistical analysis. Two postmenopausal and three premenopausal patients had centrally located tumours. Thus, benign tissue estrogen levels in the tumour-bearing breast quadrant could be compared to the non-tumour-bearing quadrants in 28 postmenopausal and 10 premenopausal women, while 30 postmenopausal and 13 pre-menopausal patients were available for the other parts of the statistical analysis. Patient demographics are given in Table 1.

Estrogen levels in benign and malignant tissue in pre- and postmenopausal women are presented in Tables 2 and 3 and Figs. 1–4. For plasma levels, the geometrical mean values for $E_2$, $E_1$ and $E_1S$ in postmenopausal women were 14.6 (95% CI 10.7–19.9), 72.4 (57.5–91.1) and 541.1 pM (378.9–773.0), respectively. As for pre-

**Table 2**
Benign breast tissue estrogen levels in tumour-bearing as compared to non-tumour-bearing breast quadrants (mean value of the three benign quadrants).

| | $E_2$ | $E_1$ | $E_1S$ |
|---|---|---|---|
| Postmenopausal women | | | |
| Tumour-bearing quadrant | 23.6 (14.3–39.1) | 489.9 (360.2–666.4) | 49.9 (28.9–86.1) |
| Non-tumour-bearing quadrants | 26.9 (16.8–42.9) | 465.1 (333.2–649.2) | 60.4 (33.0–110.7) |
| Premenopausal women | | | |
| Tumour-bearing quadrant | 450.0 (272.8–742.2) | 1143.1 (776.6–1682.7) | 385.8 (145.2–1025.4) |
| Non-tumour-bearing quadrants | 420.6 (231.5–764.0) | 1154.0 (645.1–2064.4) | 300.1 (139.4–646.0) |

Data presented as geometrical mean values (fmol/g) with 95%CI of the mean.

**Table 3**
Tissue estrogen levels in benign and malign breast samples from the same breast in correlation to menopausal status, as well as ER status (fmol/g tissue, given as geom. mean levels with 95% CI).

| ER/PGR | $E_2$ (benign) | $E_2$ (tumour) | $E_1$ (benign) | $E_1$ (tumour) | $E_1S$ (benign) | $E_1S$ (tumour) |
|---|---|---|---|---|---|---|
| Postmenopausal women | | | | | | |
| Total group ($n=30$) | 29.2 | **121.5**$^{\#\#\#}$ | 477.2 | **143.7**$^{\#\#\#}$ | 53.8 | **99.5**$^{\#\#\#}$ |
| | (19.3–44.1) | **(63.6–232.0)** | (366.7–620.9) | **(93.3–221.3)** | (32.0–90.4) | **(53.8–184.0)** |
| ER+ ($n=21$) | 31.1 | **267.1**$^{\#\#\#***}$ | 467.6 | **167.6**$^{\#\#\#}$ | 56.1 | **98.6**$^{\#}$ |
| | (19.5–49.7) | **(159.9–446.2)** | (338.2–646.4) | **(100.1–280.5)** | (29.3–107.4) | **(44.8–217.1)** |
| ER− ($n=9$) | 25.1 | 19.3 | 500.3 | **100.3**$^{(\#)}$ | 48.8 | **101.6**$^{\#}$ |
| | (9.1–69.6) | (6.1–61.5) | (285.4–876.8) | **(39.9–252.3)** | (17.0–140.5) | **(31.6–327.2)** |
| Premenopausal women | | | | | | |
| Total group ($n=13$) | 453.0 | **615.3**$^{(\#)}$ | 1233.1 | **194.6**$^{\#\#\#}$ | 394.4 | **612.6**$^{(\#)}$ |
| | (314.3–652.8) | **(230.0–1646.3)** | (817.5–1859.5) | **(109.9–344.5)** | (213.0–730.1) | **(308.6–1216.2)** |
| ER+ ($n=7$) | 398.7 | **1621.8**$^{\#**}$ | 1144.2 | **245.4**$^{\#}$ | 331.6 | 447.8 |
| | (243.2–653.4) | **(863.2–3047.9)** | (659.0–1986.6) | **(117.0–514.5)** | (113.7–967.4) | (146.2–1371.8) |
| ER− ($n=6$) | 525.9 | 198.7 | 1345.2 | **148.5**$^{\#}$ | 482.8 | **883.1**$^{(\#)}$ |
| | (253.2–1092.4) | (32.7–1207.5) | (571.5–3167.4) | **(46.7–471.6)** | (189.5–1230.3) | **(303.8–2566.8)** |

Abbreviations: n.a.: not applicable; *Statistical significant difference compared to ER− subgroup of patients; $^{**}p<0.01$; $^{***}p<0.001$ (Mann–Whitney test); #Statistical significant difference compared to benign tissue $^{(\#)}0.05<p<0.10$; $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ (Wilcoxon-Matched Pair Signed rank test).

menopausals, mean levels of plasma $E_2$, $E_1$ and $E_1S$ were 270.9 (151.1–485.7), 258.0 (189.1–352.0) and 4130.5 (2502.1–6817.1). Four of the premenopausals had plasma progesterone levels revealing the samples to be obtained during the luteal phase of the menstrual cycle. While this number is too small for statistical comparison, data do not suggest any major difference between these women and the other premenopausal individuals with respect to tissue estrogen ratios.

We compared benign tissue levels of $E_2$, $E_1$ and $E_1S$ across the four different benign breast quadrants in individual patients. The results for postmenopausal patients are shown in Fig. 1a–c. For consistency, the benign quadrants are numbered clockwise, with the tumour-bearing quadrant numbered as 1. Variation across the different quadrants among the 4 postmenopausal women receiving HRT < 1 month prior to inclusion is depicted in Fig. 2a–c. Interestingly, one of these patients expressed unusual high estrogen levels within her tumour ($E_2$: 2640 fmol/g, $E_1$: 10,890 fmol/g and $E_1S$: 7915 fmol/g), benign tissue ($E_2$: 454 fmol/g, $E_1$: 21,673 fmol/g and $E_1S$: 7657 fmol/g) as well as in plasma ($E_2$: 187 pM, $E_1$: 1970 pM and $E_1S$: 18,633 pM), consistent with a slow turn-over rate.

The coefficient of variation between the four breast quadrants was 33%, 24% and 57% with respect to $E_2$, $E_1$ and $E_1S$ in premenopausal women. Corresponding values for postmenopausals were 57%, 25% and 56%. As shown in Table 2, no difference in tissue estrogen levels between the tumour-bearing quadrant and the average level across the other breast quadrants was recorded either among premenopausal or postmenopausal women ($p>0.1$ for each comparison).

Table 4 presents correlations between tumour, benign tissue and plasma estrogen levels among pre- and postmenopausal women.

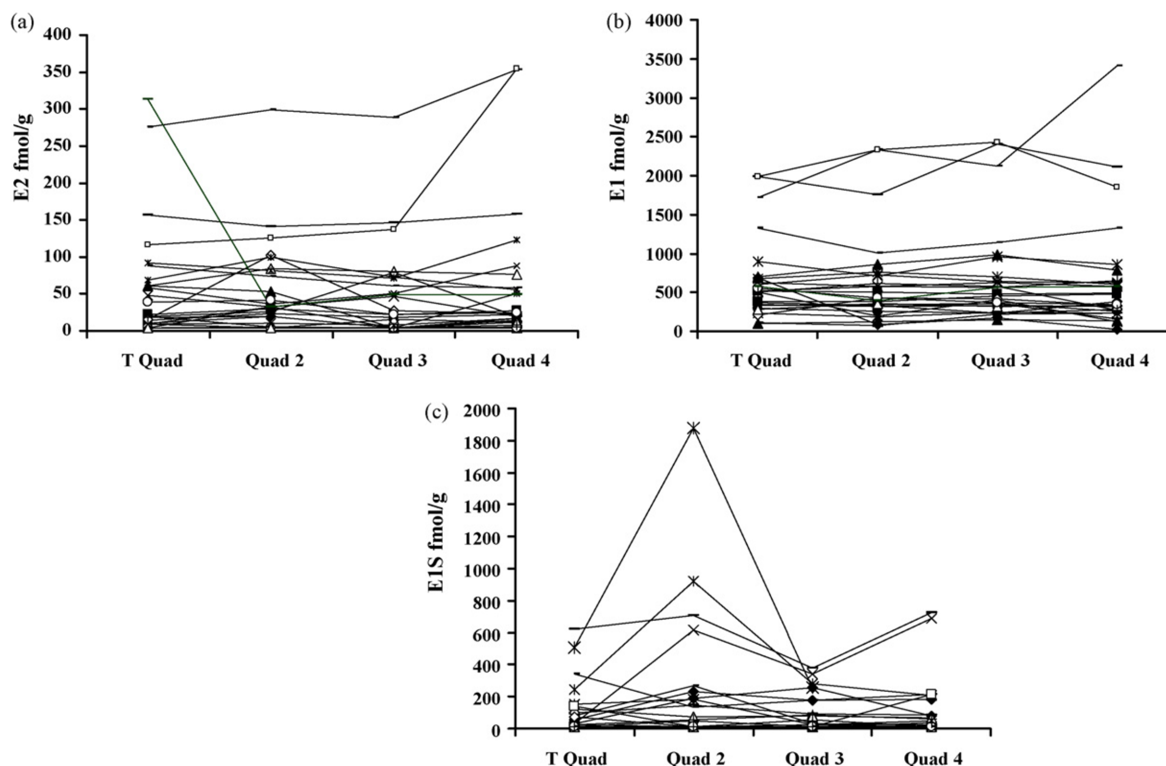


**Fig. 1.** Variation in breast quadrant tissue estrogen levels in postmenopausal women. Tumour-bearing quadrant to the left, the residual quadrants are numbered clockwise thereafter. (a) $E_2$, (b) $E_1$, (c) $E_1S$.

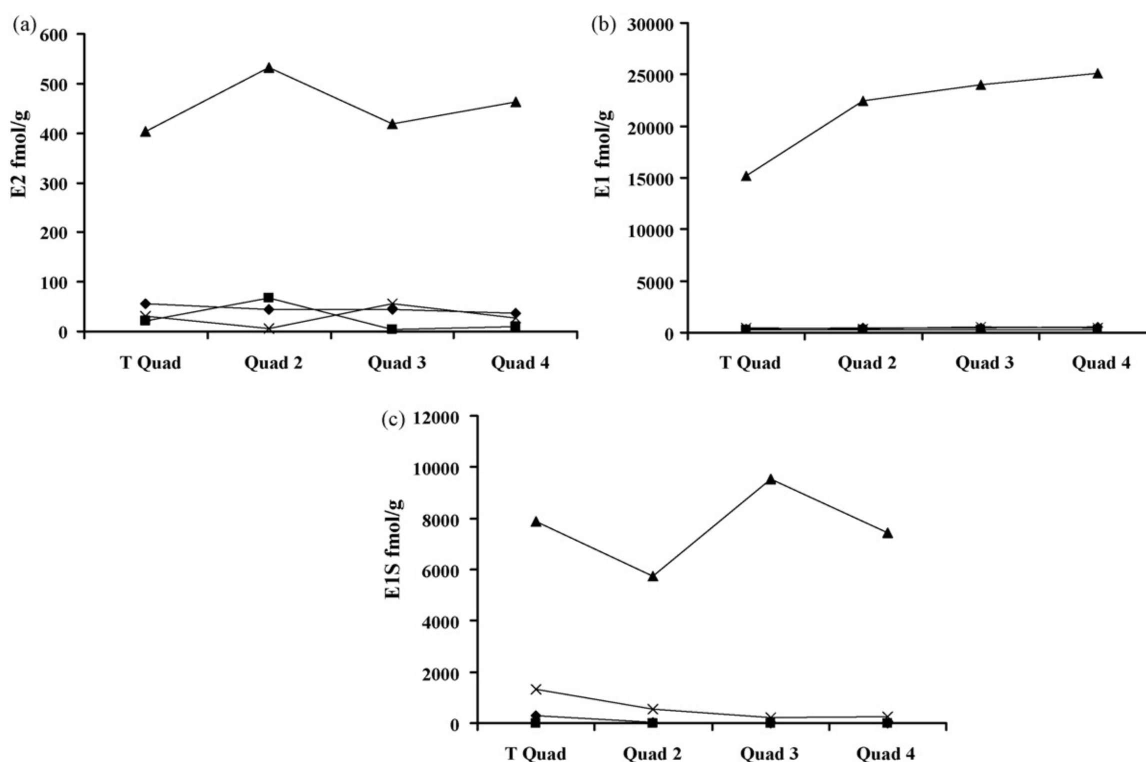


**Fig. 2.** Variation in breast quadrant tissue estrogen levels in postmenopausal women receiving HRT < 1 month prior to surgery. Tumour-bearing quadrant to the left, the residual quadrants are numbered clockwise thereafter. (a) $E_2$, (b) $E_1$, (c) $E_1S$.

**Table 4**
Correlations (Spearman's *R*) between plasma, benign and malignant hormone levels and BMI among pre- and postmenopausal breast cancer patients.

| | | TU E1 | TU $E_1$S | NT $E_2$ | NT $E_1$ | NT $E_1$S | PL $E_2$ | PL $E_1$ | PL $E_1$S | PL A | PL T | BMI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TU $E_2$ | *Postm.* | **0.393**$^{*}$ | −0.147 | 0.309 | 0.145 | −0.097 | 0.229 | **0.415**$^{*}$ | 0.173 | 0.068 | 0.309 | −0.023 |
| | *Prem.* | **0.665**$^{*}$ | 0.060 | 0.132 | 0.220 | 0.187 | 0.236 | −0.055 | −0.042 | −0.345 | −0.232 | −0.049 |
| TU $E_1$ | *Postm.* | | 0.077 | **0.364**$^{*}$ | **0.486**$^{**}$ | 0.174 | 0.328 | **0.559**$^{**}$ | 0.331 | **0.400**$^{*}$ | **0.588**$^{**}$ | 0.329 |
| | *Prem.* | | 0.319 | 0.352 | 0.368 | 0.225 | −0.042 | −0.248 | −0.212 | −0.273 | −0.369 | 0.181 |
| TU $E_1$S | *Postm.* | | | 0.283 | −0.052 | **0.850**$^{**}$ | 0.073 | −0.058 | 0.188 | 0.096 | −0.072 | −0.213 |
| | *Prem.* | | | **0.775**$^{**}$ | **0.731**$^{**}$ | **0.736**$^{**}$ | **0.685**$^{*}$ | **0.806**$^{**}$ | **0.782**$^{**}$ | 0.036 | −0.323 | −0.082 |
| NT $E_2$ | *Postm.* | | | | **0.608**$^{**}$ | 0.261 | **0.531**$^{**}$ | **0.476**$^{*}$ | **0.434**$^{*}$ | 0.013 | 0.244 | 0.381 |
| | *Prem.* | | | | **0.835**$^{**}$ | **0.571**$^{*}$ | **0.685**$^{*}$ | **0.745**$^{*}$ | **0.782**$^{**}$ | 0.009 | −0.419 | −0.220 |
| NT $E_1$ | *Postm.* | | | | | −0.093 | **0.576**$^{**}$ | **0.642**$^{**}$ | **0.608**$^{**}$ | 0.202 | **0.617**$^{**}$ | **0.592**$^{**}$ |
| | *Prem.* | | | | | 0.489 | 0.345 | **0.685**$^{*}$ | 0.552 | −0.091 | −0.433 | −0.049 |
| NT $E_1$S | *Postm.* | | | | | | 0.016 | 0.002 | 0.181 | 0.073 | −0.063 | −0.174 |
| | *Prem.* | | | | | | **0.636**$^{*}$ | **0.648**$^{*}$ | **0.685**$^{*}$ | −0.082 | −0.164 | 0.027 |
| PL $E_2$ | *Postm.* | | | | | | | **0.864**$^{**}$ | **0.640**$^{**}$ | 0.277 | **0.576**$^{**}$ | 0.177 |
| | *Prem.* | | | | | | | **0.806**$^{**}$ | **0.770**$^{**}$ | 0.261 | 0.073 | −0.248 |
| PL $E_1$ | *Postm.* | | | | | | | | **0.680**$^{**}$ | 0.225 | **0.684**$^{**}$ | 0.141 |
| | *Prem.* | | | | | | | | **0.842**$^{**}$ | 0.333 | 0.036 | −0.309 |
| PL $E_1$S | *Postm.* | | | | | | | | | 0.043 | **0.550**$^{**}$ | 0.116 |
| | *Prem.* | | | | | | | | | 0.139 | −0.310 | −0.600 |
| PL A | *Postm.* | | | | | | | | | | **0.528**$^{**}$ | 0.080 |
| | *Prem.* | | | | | | | | | | **0.688**$^{**}$ | 0.064 |
| PL T | *Postm.* | | | | | | | | | | | 0.204 |
| | *Prem.* | | | | | | | | | | | 0.292 |

$^{*}$ $p \le 0.05$ (two-tailed).
$^{**}$ $p \le 0.01$ (two-tailed).

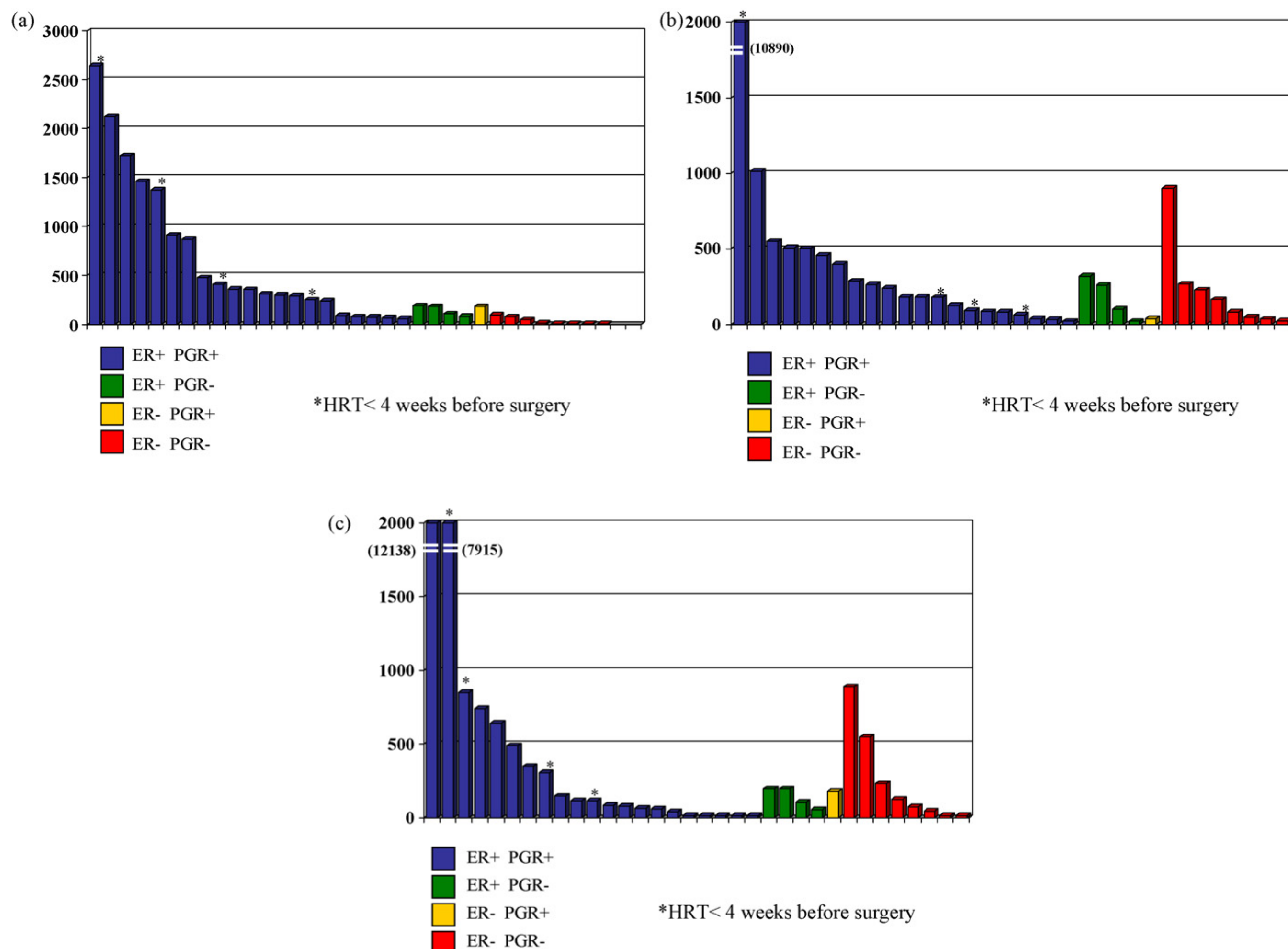


**Fig. 3.** Intratumoural concentrations in fmol/g of $E_2$, $E_1$ and $E_1S$ related to expression of the ER and PgR in tumours from postmenopausal women. (a) $E_2$, (b) $E_1$, (c) $E_1S$.

For benign tissue levels, we used the mean value of the four different quadrants for statistical analysis. Tumour $E_2$ as well as $E_1$ correlated weakly to normal tissue levels, contrasting tumour $E_1S$ levels, which strongly correlated to normal tissue $E_1S$ levels among pre- as well as postmenopausal patients. Considering intratumour estrogen levels, tumour $E_2$ and $E_1$ levels correlated weakly in pre- as well as postmenopausal women. In contrast, no correlation between tumour $E_1S$ and either $E_1$ or $E_2$ levels was recorded. Evaluation of the ER positive and ER negative subgroups separately provided similar results (Table 5).

Normal tissue $E_2$ as well as $E_1$ levels in general correlated significantly to plasma estrogen levels. A correlation in-between the different plasma estrogen fractions are in accordance with previous findings from our group [26]. While plasma $E_2$, $E_1$ and $E_1S$ each correlated to normal tissue $E_1$ and $E_2$ levels, a correlation to normal tissue $E_1S$ was observed among premenopausal women only. While a positive correlation between plasma levels of testosterone and each of the three plasma estrogen fractions in postmenopausal women and between plasma T and A levels in pre- as well as in postmenopausal women were recorded, no correlation between plasma androgen levels and any of the tissue estrogens fractions was observed, except for a weak correlation to tumour $E_1$ in postmenopausal women.

Individual intratumour estrogen values related to ER as well as PgR status are depicted in Figs. 3a–c and 4a–c. Contrasting our expectations, tumour $E_1$ levels were suppressed compared to benign tissue levels among ER+ and ER− tumours in pre- (average ratio $E_1$ cancer versus benign tissue 0.2; $p < 0.001$) as well as in postmenopausal (average ratio $E_1$ cancer versus benign tissue 0.3; $p < 0.001$) women (Fig. 5 and Table 6). Most interestingly, $E_2$ levels in ER positive tumours were significantly higher compared to $E_2$ levels in benign tissue in pre- (average ratio: 4.1; $p < 0.05$) as well as in postmenopausal (average ratio 8.6; $p < 0.001$) women (Fig. 5). No difference in $E_2$ levels between ER negative tumours and benign tissue was recorded. Thus, tumour $E_2$ levels were significantly higher among ER+ compared to ER− tumours (Table 2 and Figs. 3a–c and 4a–c; $p < 0.01$ with respect to pre- as well as postmenopausal women when analyzed separately). Notably, $E_1S$ was significantly higher in tumour compared to benign tissue for ER positive as well as ER negative tumours in postmenopausal patients (total group: $p < 0.001$; $p < 0.05$ for each group evaluated separately).

Finally, we compared benign tissue and plasma estrogen levels between patients with ER+ and ER− tumours to address whether high benign tissue/and/or plasma estrogen values may be associated with ER+ tumours in particular. No difference with respect to any benign tissue or plasma estrogen fraction between patients expressing ER+ or ER− tumours was recorded. Interestingly, BMI revealed a significant correlation to tissue but not to plasma $E_2$ and $E_1$ levels.

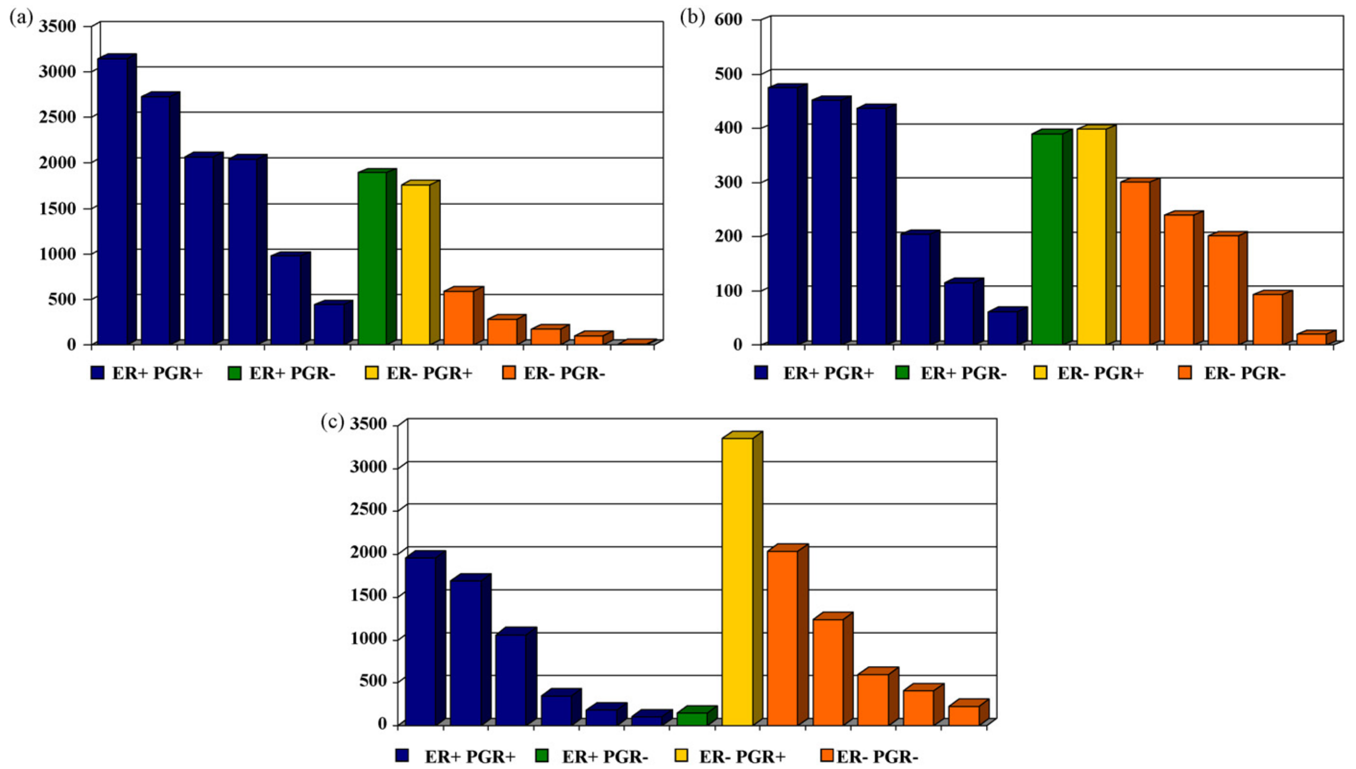


**Fig. 4.** Intratumoural concentrations in fmol/g of $E_2$, $E_1$ and $E_1S$ related to expression of the ER and PgR in tumours from premenopausal women. (a) $E_2$, (b) $E_1$, (c) $E_1S$.

**Table 5**
Correlations (Spearman's R) between plasma, benign and malignant hormone levels and BMI among postmenopausal.

| | | TU E1 | TU $E_1$S | NT $E_2$ | NT $E_1$ | NT $E_1$S | PL $E_2$ | PL $E_1$ | PL $E_1$S | PL A | PL T | BMI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TU $E_2$ | *ER+* | **0.461**[*] | −0.362 | **0.434**[*] | **0.452**[*] | −0.379 | **0.549**[*] | **0.597**[**] | 0.150 | 0.360 | **0.578**[**] | 0.190 |
| | *ER−* | −0.051 | 0.604 | 0.034 | −0.542 | 0.638 | −0.415 | −0.390 | −0.415 | −0.244 | −0.659 | −0.373 |
| TU $E_1$ | *ER+* | | 0.093 | **0.587**[**] | **0.613**[**] | 0.212 | **0.662**[**] | **0.774**[**] | **0.533**[*] | **0.493**[*] | **0.675**[**] | **0.527**[*] |
| | *ER−* | | 0.293 | 0.100 | 0.267 | 0.373 | −0.190 | −0.143 | −0.524 | 0.143 | 0.143 | 0.233 |
| TU $E_1$S | *ER+* | | | 0.258 | 0.022 | **0.796**[**] | 0.085 | −0.006 | 0.279 | −0.034 | 0.042 | −0.105 |
| | *ER−* | | | 0.293 | −0.234 | **0.987**[**] | −0.048 | −0.216 | −0.012 | 0.311 | −0.443 | −0.276 |
| NT $E_2$ | *ER+* | | | | **0.605**[**] | 0.285 | **0.624**[**] | **0.665**[**] | **0.472**[*] | 0.123 | **0.509**[*] | 0.102 |
| | *ER−* | | | | 0.600 | 0.237 | 0.310 | −0.262 | 0.000 | −0.095 | −0.405 | 0.583 |
| NT $E_1$ | *ER+* | | | | | 0.020 | **0.612**[**] | **0.702**[**] | **0.725**[**] | 0.237 | **0.679**[**] | 0.276 |
| | *ER−* | | | | | −0.237 | 0.548 | 0.357 | 0.238 | 0.024 | 0.476 | **0.900**[**] |
| NT $E_1$S | *ER+* | | | | | | 0.050 | 0.048 | 0.276 | 0.002 | 0.057 | −0.107 |
| | *ER−* | | | | | | −0.073 | −0.195 | −0.146 | 0.317 | −0.415 | −0.237 |
| PL $E_2$ | *ER+* | | | | | | | **0.928**[**] | **0.711**[**] | 0.327 | **0.789**[**] | 0.139 |
| | *ER−* | | | | | | | **0.810**[*] | 0.476 | 0.071 | 0.048 | 0.476 |
| PL $E_1$ | *ER+* | | | | | | | | **0.749**[**] | 0.302 | **0.790**[**] | 0.159 |
| | *ER−* | | | | | | | | 0.429 | 0.167 | 0.333 | 0.238 |
| PL $E_1$S | *ER+* | | | | | | | | | 0.053 | **0.649**[**] | 0.311 |
| | *ER−* | | | | | | | | | 0.119 | 0.310 | −0.071 |
| PL A | *ER+* | | | | | | | | | | **0.493**[*] | 0.490 |
| | *ER−* | | | | | | | | | | 0.548 | −0.143 |
| PL T | *ER+* | | | | | | | | | | | 0.172 |
| | *ER−* | | | | | | | | | | | 0.286 |

ER+ and ER− patients.
[*] $p \le 0.05$ (two-tailed).
[**] $p \le 0.01$ (two-tailed).

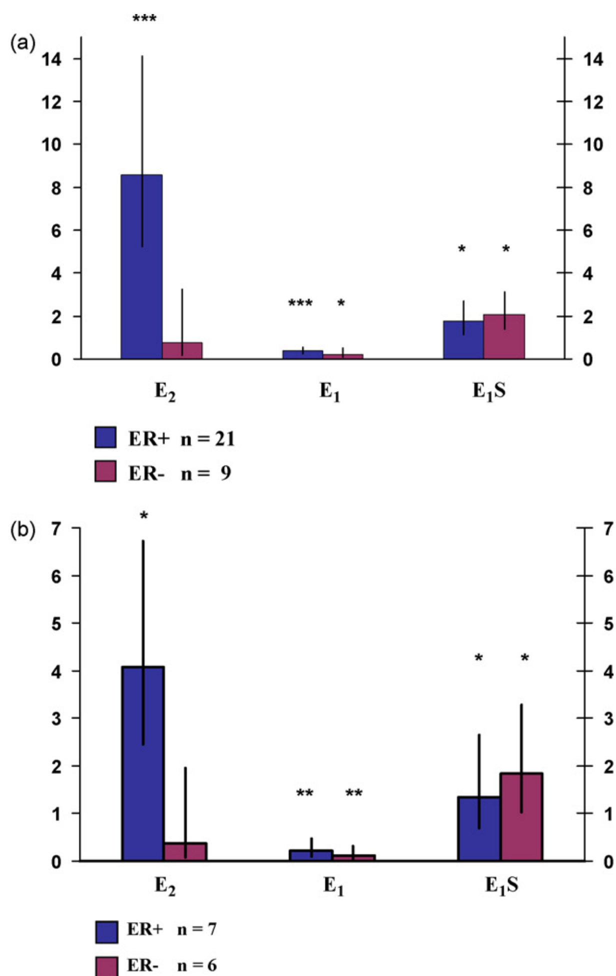


**Fig. 5.** Ratio between intratumour and benign breast tissue estrogen levels related to estrogen receptor expression and menopausal status (a) postmenopausal women, (b) premenopausal women. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

**Table 6**
Ratios of diverse plasma and tissue estrogen fractions in subgroups of patients; geom. mean (with 95% CI).

| ER-status | Postmenopausal women ER+ n = 21 | Postmenopausal women ER− n = 9 | Premenopausal women ER+ n = 7 | Premenopausal women ER− n = 6 |
|---|---|---|---|---|
| TU-$E_2$/TU-$E_1$ | **1.5**[**] **(0.9–2.6)** | 0.2 (0.0–1.1) | **7.1**[*] **(4.5–11.3)** | 0.8 (0.1–8.5) |
| TU-$E_2$/PL-$E_2$ | **16.4**[***] **(10.8–24.9)** | 1.2 (0.3–5.3) | **6.6**[*] **(2.2–20.5)** | 0.5 (0.0–9.7) |
| TU-$E_1$/PL-$E_1$ | 2.1 (1.5–3.0) | 1.4 (0.5–4.2) | 1.0 (0.3–2.7) | 0.7 (0.2–2.6) |
| TU-$E_1$S/PL-$E_1$S | 0.2 (0.1–0.4) | 0.2 (0.0–0.8) | 0.1 (0.0–0.3) | 0.2 (0.1–0.6) |
| NT-$E_2$/PL-$E_2$ | 2.0 (1.3–3.2) | 1.3 (0.5–3.2) | 1.6 (0.7–3.5) | 2.2 (1.7–3.0) |
| NT$E_1$/PL-$E_1$ | 5.9 (5.0–7.0) | 6.3 (4.4–9.0) | 4.4 (2.8–6.9) | 7.4 (4.7–11.5) |
| NT$E_1$S/PL-$E_1$S | 0.1 (0.0–0.2) | 0.1 (0.0–0.4) | 0.1 (0.0–0.3) | 0.1 (0.0–0.3) |

Abbreviations: ER, estrogen receptor; $E_1$, estrone; $E_2$, estradiol; $E_1$S, estrone sulphate; TU, tumour tissue; NT, normal (benign) tissue of the same breast; PL, plasma; statistical significant difference compared to ER− subgroup of patients.
[*] $p \leq 0.05$.
[**] $p \leq 0.01$.
[***] $p \leq 0.001$ (Mann–Whitney test).

## 4. Discussion

Estradiol has a profound effect on breast cancer development and growth. Thus, plasma estrogen levels [1] as well as normal breast mammographic density [23] are both strongly associated with subsequent risk for breast cancer development, and translational studies on aromatase inhibitors have revealed a correlation between degree of estrogen suppression and clinical outcome [3,28] but also endogenous estrogen levels and time to progression in breast cancers [29]. Contrasting the fact that the enzymes as well as organ systems involved in postmenopausal estrogen synthesis have been known for decades [4] and the effect of different ligands with respect to expression of the aromatase enzyme extensively studied *in vitro* [24,30], we do not know which factors that may be of importance regulating estrogen synthesis either in benign or breast cancer tissue.

The finding that intratumour $E_2$ concentration may exceed plasma concentration, in particular among ER+ tumours [5–14], has focused on the potential of local estrogen production being a main contributor to intratumour hormone levels. Yet, results so far with respect to local versus systemic contribution have been restricted to a limited number of individuals, providing conflicting results [31,32].

A limiting factor studying these mechanisms has been the need for sensitive methods assessing plasma as well as tissue estrogen levels in postmenopausal women. This issue in particular has been highlighted when studying endocrine effects of aromatase inhibitors [3]. Although tracer studies revealed first- and second-generation aromatase inhibitors as aminoglutethimide [33,34], fadrozole [35] and formestane [36] to inhibit *in vivo* total body aromatization by >85%, estrogen measurement revealed plasma level suppression of $E_2$ and $E_1$ of about 50% with these compounds only [37–40]. This lead us [16,25,27] as well as others [41,42] to invest much efforts into developing highly sensitive assays for plasma as well as tissue estrogen measurement. In our assays, we converted $E_1$, as well as $E_1$S into $E_2$, allowing final detection with a highly specific antibody. Moreover, each sample went through pre-analytical purification steps as described in detail. In addition to formal method validations [16,25], using these assays we detected plasma [43,44] and tissue [45,46] estrogen suppression approaching the percentage suppression detected in tracer studies, contrasting what was shown with use of earlier radioimmunoassay. Furthermore, these methods were able to discriminate plasma [44] as well as tissue [46] estrogen suppression between the potent third-generation aromatase inhibitors anastrozole and letrozole.

While others have reported use of an ultrasensitive yeast bioassay [47] or GC/MS/MS [48] to assess estrogen suppression in postmenopausal women, these methods have been validated with respect to plasma $E_2$ only. Considering the bioassay, calculations based on androgen plasma levels, percentage aromatization and estrogen clearance rates have suggested the values reported to be lower than expected [49], contrasting the average levels of $E_2$ and $E_1$ recorded with our radioimmunoassay that fits well to theoretical expectations. While the GC/MS/MS method [48] revealed superiority compared to radioimmunoassay measuring estrogen concentrations in the low range, notably none of the contemporary radioimmunoassays, including our method, was included for comparison.

Considering postmenopausal plasma estrogen levels, the values reported here are in the same range as previously reported from our group [50] as well as others [42] using highly sensitive radioimmunoassay. These values in general are somewhat lower compared to results previously reported due to improved method sensitivity. As for tissue estrogen levels, they are consistent with previous findings in our laboratory [45,46]. Again, these values are somewhat

lower compared to previous results achieved with use of radioimmunoassay not involving similar pre-analytical purification steps [51,52].

In this study, we measured elevated levels of $E_1$ as well as $E_2$ but not $E_1S$ in benign tissue compared to plasma. In postmenopausal women, estrogens in general are synthesized in most body compartments. As such, plasma estrogen levels arise by simple diffusion into plasma from the tissue compartments [3]. Considering plasma clearance rate of $E_1$ and $E_2$ to be in the range of 30–50 L/h, contrasting a slow clearance rate of 5–8 L/h for $E_1S$ [38,53], this may explain the finding of a high concentration ratio between tissue and plasma levels of $E_2$ and $E_1$ but why plasma $E_1S$ levels remain high.

A seminal finding was the result by Professor Millers group two decades ago revealing elevated estrogen synthesis in the tumour-bearing breast quadrant [12]. Interestingly, we did not observe increased estrogen levels in the tumour-bearing quadrant. This observation, together with the finding of reduced levels of intratumour $E_1$, does not suggest locally elevated estrogen production, due to example inflammatory processes enhancing aromatase activity [54], to play a significant role as a tumour growth stimulator.

The potential contribution from local versus systemic estrogens to intratumoural estrogen levels remains an issue of controversy. Intratumoural estrogens may be delivered through the circulation, in which case it is synthesized in body compartments in general [4]. Alternatively, considering breast density may exceed plasma estrogen levels as a risk factor to breast cancer development [23], some women may have a propensity for elevated estrogen synthesis in the breast tissue. This hypothesis is supported by data revealing a strong heritability with respect to breast density [55]. Notably, looking at pre- as well as postmenopausal populations, we found a strong correlation between benign breast tissue and plasma estrogen levels. Further, we recorded weaker correlations between benign tissue as well as plasma estrogen levels on the one hand and intratumoural levels on the other hand. We suggest the following interpretation to these findings: First, plasma estrogens are synthesized in most normal body tissues, from which they passively leak into the plasma. Plasma estrogen levels are subject to inter-individual variation with respect to metabolism [38], weakening the direct correlation between plasma and benign tissue levels. As for intratumour estrogen levels, except for intratumour $E_1S$, that seems to mirror benign tissue levels, $E_1$ and $E_2$ levels are likely influenced by local effects on disposition. The correlation between intratumour and benign tissue estrogen levels observed may be due either to direct uptake of estrogens from benign tissue into the tumour or, alternatively, the fact that intratumour and benign tissue estrogens are subject to similar control mechanisms (genetic polymorphisms affecting intra- as well as extra-tumoural enzyme activity). Further studies are warranted to address this issue.

While we observed no correlation between plasma androgen and tissue estrogen levels (except for a weak correlation to tumour $E_1$ in the postmenopausal women), these results should be interpreted carefully due to uncertainties related to low androgen levels detected in postmenopausal women.

A most striking observation was the finding of reduced intratumour level of $E_1$ across all tumour categories. There may be several reasons for these observations. One explanation involves enhanced conversion of $E_1$ into $E_2$. Estrone and $E_2$ are inter-convertible through a panel of 17beta-hydroxysteroid dehydrogenases (17-HSDs). While the 17beta-HSD isoforms B1, 2, 7 and 12 mainly express reductive activity catalyzing the activation of $E_1$ into $E_2$ [56–59], isoforms like B2, 12 and 14 catalyze oxidation of $E_2$ into $E_1$ [60–62]. Thus, either an increase in reduction capacity or reduced oxidation capacity may alter this balance. This explanation is consistent with the finding of elevated $E_2$ levels in ER+ tumours, but are less consistent with a low concentration of $E_2$ in ER− ones. Our second key observation was the finding of elevated $E_2$ levels in ER+ tumours when compared to levels in benign tissue as well as when compared to the levels in ER− tumours. This was confirmed by an elevated tumour to benign tissue ratio for $E_2$ as well as an increased intratumour $E_2$ to $E_1$ ratio among patients with ER+ tumours.

A second explanation to the high $E_2$ level may be enhanced local estrogen synthesis in ER+ tumours. However, if that was the main explanation, we should anticipate tumour $E_1$ to be elevated, considering aromatization of androstenedione (A) into $E_1$ to be the main pathway of estrogen synthesis [4].

A third explanation to elevated $E_2$ levels in ER+ tumours involves enhanced ligand binding capacity for $E_2$. While contemporary studies evaluating expression of the estrogen receptor in general use immunohistochemistry, three decades ago the general methods were ligand binding assays [63]. Thus, in a large study summarising data from nearly 2000 individual tumours, Harvey et al. reported average estrogen receptor concentration among receptor positive tumours to be about 100–150 fmol/mg protein [64]. Assuming a protein concentration of approximately 70 mg/g tissue, 100 fmol/mg protein corresponds to about 7 pmol/g tissue. Thus, even in tumours expressing high levels of $E_2$, most of the ER remains unoccupied by its ligand.

While the tissue estrogen levels presented here on average are lower compared to previous studies conducted one or two decades ago, they are in accordance with previous results reported by our group [45,46]. The reason why we detect lower levels compared to previous studies most likely is due to the extensive purification steps involved in this method prior to RIA detection and use of a highly specific antibody, thus avoiding non-specific interactions.

Finally, we compared plasma and benign tissue estrogen levels among patients harbouring ER+ versus ER− tumours. The finding of no difference in estrogen levels between the two groups should be interpreted carefully, considering the limited number of observations. On the other hand, there may be biological explanations to these findings. While plasma levels of $E_2$ have been found correlated to an increased risk of breast cancer in postmenopausal women [65], such an hormonal effect may occur at an early stage of sub-clinical cancer development. Further evidence supporting a role of estrogen stimulation in early carcinogenesis with respect to ER− tumours is provided by the fact that ovarian suppression may reduce breast cancer risk among patients carrying a *BRCA1* mutation [66], in which case most of the tumours are hormone receptor negative.

Obesity has been linked to postmenopausal breast cancer risk [67] as well as breast cancer prognosis [68]. Previously, we recorded a moderate correlation between plasma estrogen levels and BMI [26]. Here, this association was significant with respect to tissue estrogen concentrations only. Notably, there is evidence obesity may enhance cellular aromatase activity in fibroblasts [69]. The finding of a weaker association between plasma estrogen levels compared to BMI may be due to individual variations with respect to estrogen metabolism as indicated above.

In conclusion, this paper provides novel information adding to our understanding of intratumour estrogen dynamics. The finding that intratumour $E_1$ levels were consistently lowered compared to benign breast tissue concentrations argues against the hypothesis that breast cancer estrogens are produced by intratumoural aromatization. In contrast, a significant increase in tumour $E_2$ when compared to benign tissue as well as to ER− tumour tissue indicates alternative mechanisms as receptor ligand binding or enhanced enzymatic conversion of $E_1$ into $E_2$ by the 17beta-HSD system to explain elevated $E_2$ levels in ER+ breast cancer tissue. Further investigations should be directed exploring these mechanisms which may have significant therapeutic implications.

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