Tissue Content of Hydroxyestrogens in Relation to Survival of Breast Cancer Patients[1]

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ABSTRACT

Purpose: **The main goal of our study was to assess estrogen contents of breast tumor tissues, having different estrogen receptor status, in relation to long-term follow-up of patients.**

Experimental Design: **Twenty-one breast cancer cases, all collected from January 1986 to January 1988 at the M. Ascoli Cancer Hospital Centre in Palermo, were included in the study and compared with 6 healthy women as a control group. Average follow-up time of patients was 144 ± 10 months. The estrogen receptor status of tissues was determined by both ligand binding and immunohistochemical assays. A high performance liquid chromatography-based approach, jointly with gas chromatography/mass spectrometry, was used to identify and measure main estrogens, various hydroxyestrogens, and their methoxy derivatives in both normal and tumor tissues.**

Results: **Although variable concentrations of hydroxylated estrogens were detected, they consistently accounted for >80% of all of the estrogens. Significantly greater amounts of both 2- and 4-hydroxyestradiol, along with a marked increase of 16α-hydroxyestrone (OHE_1), were observed in cancer with respect to normal breast tissues. A significant positive association was observed with elevated $16\alpha OHE_1$ ($P = 0.015$) in patients alive, leading to significantly lower ($P = 0.043$) $2OHE_1$:$16\alpha OHE_1$ ratio values. Conversely, ratio values of 4:2 hydroxy+methoxy estrogens was significantly lower ($P = 0.006$) in deceased patients. Using cutoff values of 1.2 for 4:2 hydroxy+methoxy ratio and 150 fmol/mg tissue for $16\alpha OHE_1$ we achieved a clear-cut separation of patients, with over-cutoff patients having 147 months and under cutoff patients showing only 47 months median survival time ($P = 0.00008$).**

Conclusions: **Our data imply that individual hydroxyestrogens may have a distinct role in the onset and the clinical progression of breast cancer, with greater $16\alpha OHE_1$ levels being in turn associated to cancer with respect to normal tissues and to a prolonged survival of breast cancer patients.**

INTRODUCTION

The implication of estrogens in development of tumors at various organ sites of both animals and humans has been universally recognized over the last 70 years. In mammary carcinogenesis, recent evidence supports a dual role for the natural hormone E_2[3] as a stimulator of cell proliferation and as a procarcinogen inducing DNA damage; in this respect, local formation of estrogen derivatives and their metabolic activation to reactive intermediates may play a major role in both processes (reviewed in Ref. 1).

In the human breast, interconversion of main estrogens, E_2 and E_1, is commonly followed by two major pathways, which consist of hydroxylation at the C-16α or at C-2/C-4 positions, to respectively yield $16\alpha OHE_1$ on one hand, or the CCE, namely the 2-OH and 4-OH estrogens, on the other. The former may be additionally converted to E_3, the latter can also be metabolized by catechol-*O*-methyltransferase to their MeO derivatives. The concept that estrogens, likewise testosterone in the human prostate, need to be converted to biologically active derivatives for full expression of hormone activity was first introduced by Fishman and Norton (2). The authors suggested that 2-hydroxylated estrogens may mediate the action of the hormone parent, having properties distinct but relevant to the physiology or pathophysiology of a target organ. Today, it is recognized that CCE can be generated by at least three distinct microsomal, P450-mediated enzymatic activities, namely 2-, 4-, and 2/4-hydroxylases, which produce both 2- and 4-OH derivatives of either E_2 or E_1. These hydroxylase enzymes appear to be independently expressed and differentially regulated in several target tissues (3). Whereas the estrogenic potency appears to be reduced by 2-hydroxylation, it may be preserved or even enhanced by hydroxylation at the C-4 position. This could be also

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[3] The abbreviations used are: E_2, 17β-estradiol; E_1, estrone; CCE, catecholestrogen; E_3, estriol; OHE_2, hydroxyestradiol; ER, estrogen receptor; HPLC, high-performance liquid chromatography; LBA, ligand binding assay; ICA, immunohistochemical assay; PR, progesterone receptor; GC/MS, gas chromatography-mass spectrometry; RP, reversed-phase; DFS, disease-free survival; OS, overall survival; OHE_1, hydroxyestrone; MeO, methoxy; OH, hydroxy.

Table 1 Logistic regression output in terms of odds ratio (ORs) and confidence intervals (CIs)

				Univariate analysis (unadjusted)			Multivariate analysis (adjusted)		
	Dead	Alive	Odds on death	OR relative to baseline	95% CI on OR	Two-sided *P* for trend[a]	OR relative to baseline	95% CI on OR	Two-sided *P* for trend[a]
Age									
<42 Years	1	3	0.33	1.00[b]			1.00[b]		
42–51	4	2	2.00	6.00	(0.35–101.57)		4.75	(0.14–162.73)	
52–61	3	0	3.00	0.00	(0.00–0.00)		0.00	(0.00–0.00)	
>62	3	1	3.00	9.00	(0.37–220.93)	0.54	12.05	(0.36–405.56)	0.56
Menopause									
Pre	3	6	0.50	1.00[b]			1.00[b]		
Post	5	3	1.67	3.33	(0.45–24.44)	0.24	2.73	(0.30–24.79)	0.37
ER status[c]									
Positive	4	6	0.67	1.00[b]			1.00[b]		
Negative	4	3	1.33	2.00	(0.28–14.20)	0.49	2.25	(0.24–21.19)	0.48
T									
T2	3	6	0.50	1.00[b]			1.00[b]		
T3	1	2	0.50	1.00	(0.63–15.99)		1.14	(0.02–76.31)	
T4	4	1	4.00	8.00	(0.60–106.93)	0.26	6.22	(0.24–161.43)	0.48
N									
N0	1	2	0.50	1.00[b]			1.00[b]		
N1	7	7	1.00	2.00	(0.15–27.44)	0.60	2.29	(0.11–47.98)	0.59
G									
G2	5	8	0.63	1.00[b]			1.00[b]		
G3	3	1	3.00	4.80	(0.38–59.89)	0.22	4.18	(0.25–70.27)	0.32

[a] Mantel's test for trend.
[b] Referent. T, tumor size; N, nodal status; G, tumor grading.
[c] ER-positive status aggregates ER +/+ and −/+ tumors; ER-negative status includes ER −/− tumors only.

ascribed to the slower rate of dissociation of $4OHE_2$ from the ER, although the biological significance of such a prolonged association remains to be clarified.

It is widely recognized that estrogens play a primary role in development and growth of human breast cancer. Estrogen implication in mammary tumor promotion and progression has been based mostly on evidence for ER-mediated mitogenic effects (4, 5). However, the assumption that estrogens may also have a direct role as tumor initiators has been supported by the evidence that estradiol, CCE, and/or their corresponding quinone derivatives may be genotoxic both *in vitro* and *in vivo*, eventually leading to various types of DNA damage, including single-strand breaks and stable or depurinating DNA adducts (6–8).

Early studies have attempted to correlate plasma levels of circulating estrogens with breast cancer; in particular, it has been postulated that an increase in the biologically active estrogen fraction, including either free or some protein-bound estrogen, may be important for the onset of breast tumors (9, 10). In such research, results obtained have been largely at variance, and only small, although statistically significant differences between normal subjects and breast cancer patients or a modest increase of breast cancer risk have been, in turn, reported (11, 12). More recently, some cohort studies have, however, revealed a strong relationship between endogenous estrogen levels and breast cancer risk (13, 14). It ought to be emphasized that intratissue levels of estrogens in human breast have been reported to be several times (10 to 100) greater than the respective plasma values and that either normal or malignant breast tissues appear endowed with key enzyme activities of steroid metabolism (15–18). Therefore, circulating levels of individual estrogens could not by far be considered representative of the actual amounts of bioavailable estrogen to the tumor cells.

In the present work, we have adopted an original HPLC-based approach aiming to assess intratissue contents of classical estrogens (E_2, E_1, and E_3), hydroxyestrogens, and their MeO derivatives in a selection of human breast tumors, having distinct ER status and long follow-up time. To our knowledge, this is the first report ever relating intratumoral levels of individual estrogen metabolites to both clinical course of breast cancer patients and biochemical features of mammary tumor tissues. Results from these studies are herein reported.

PATIENTS AND METHODS

Subjects and Tissue Specimens. Breast tumor tissues were obtained from a series of 21 consecutive breast cancer patients, all collected at the M. Ascoli Cancer Hospital Centre in Palermo from January 1986 to January 1988, who underwent radical mastectomy for T_2-T_4 tumors. The study was approved by the Ethical Committee of M. Ascoli Cancer Centre. Informed consent was obtained from all of the patients included in the study. Some subject characteristics are summarized in Table 1. All of the patients underwent radical (Halstead or Patey-modified) mastectomy. At presentation, 3 patients had bone metastatic disease and were therefore excluded from statistical analysis and comparisons of patient survival. The mean age of patients at diagnosis was 52 ± 11 years (range, 35–71). Equivalent proportions of subjects were allocated according to menopausal and ER status, whereas most patients had involved nodes (76.2%) and G_2 tumors (66.7%). After surgery, all of the patients having nodal involvement received cyclophosphamide,

methotrexate, 5-fluorouracil (12 courses) alone; only 3 patients whose tumors were ER-positive received a combination of CMF and tamoxifen (30 mg daily). Patients were followed up at regular intervals for a mean time of 144 ± 10 months. Six histologically normal breast tissue samples were obtained from healthy women (age range 35–63 years) who underwent surgery for reduction mammoplasty. After pathological examination confirmed the absence of any incidental dysplastic or cancerous lesion, the samples were used as a control group for comparison with breast cancer patients.

Once collected, tissue specimens were carefully dissected to remove fat or fibrous tissue, weighed, and: (*a*) processed immediately for analysis of intratissue estrogens; (*b*) stored for a limited period of time (2–3 weeks) in a sucrose-glycerol buffer at −20°C until LBA of ERs; or (*c*) paraffin-embedded to obtain tissue section for immunohistochemical studies.

LBA of ERs. ER content and status of breast tumor tissues was determined by radioligand binding assay, as extensively described elsewhere (19, 20). Briefly, tissue homogenates were spun at 800 × *g* for 5 min at 4°C to separate the soluble (supernatant) from the nuclear (pellet) cell fraction. Therefore, aliquots (150 μl) of each cell fraction were incubated overnight at 4°C against increasing concentrations (from 0.1 to 5 nM) of [2,4,6,7–3H]E_2 as radioligand. For competition studies, a 100-fold excess of unlabeled diethylstilbestrol was used. After incubation, dextran-coated charcoal absorption and filtration methods were used to separate bound from unbound ligand in soluble and nuclear fraction, respectively. For the soluble fraction, samples were centrifuged at 3000 × *g* for 5 min at 4°C and 1-ml aliquots of the resulting supernatant counted in a β-counter (Beckman). For the nuclear fraction, 100-μl aliquots of the nuclear suspension were filtered through Whatman GF/C glass fiber filters (Whatman Ltd., Maidstone, United Kingdom), the unbound ligand being washed out using saline. Filters were then removed, dried overnight at room temperature, and counted up for radioactivity. Receptor data from both saturation and competition studies were analyzed and processed using Scatchard analysis and a modification (OncoLog 2.2) of a least-square fit routine (21), run on an IBM-PC, yielding both dissociation constant and concentration values (fmol/ml homogenate); the latter were expressed either as fmol/mg protein or fmol/mg DNA for soluble fraction and as fmol/mg DNA for nuclear fraction. Data were also analyzed using a model for one or two binding sites, depending on the best fitting achieved. Protein and DNA cell contents were determined using the Bradford (22) and the modified Burton (23) methods, respectively. Tumors were designated +/+ or −/+, respectively, when soluble and nuclear fractions or nuclear fraction only were ER-positive, and −/− if both cell fractions were receptor-negative.

ICA of ERs and PRs. Presence of both ERs and PRs was investigated in breast tumor tissues using an immunohistochemical ER/PR detection system (DAKO Corporation, Carpinteria, CA). This system identifies the classical ERα only, whereas it does not detect the presence of the recently discovered ERβ. Briefly, 4–6-μm thick sections of paraffin-embedded tissues were placed on microscope slides. Deparaffinization of slides was then achieved by 3 × 15 min incubation in xylene. Slides were then rehydrated through 5-min sequential dips in alcohol and rinsed in distilled water for 5 min. For antigen retrieval, slides were placed in a DAKO Target Retrieval Solution, heated three times for 4 min in a microwave oven at 750W, and finally cooled down to room temperature for 20 min. After two rinses in PBS, slides were incubated 5 min with hydrogen peroxide to block endogenous peroxidase. Slides were gently rinsed in distilled water and in PBS to remove hydrogen peroxide excess, then incubated with 10% horse serum for 10 min at room temperature to block nonspecific binding and then exposed for 1 h at room temperature in a humidified chamber to primary mouse monoclonal anti-ER or anti-PR antibodies (2 μg/ml). Parallel control slides (negative controls) were incubated using 2 μg/ml normal mouse IgG under identical conditions. After 10-min washes in PBS, slides were incubated in a humidified chamber at room temperature for 10 min with a biotinylated universal secondary antibody (Vector Laboratories, Burlingame, CA). Slides were washed two to three times for 5 min in PBS and incubated for an additional 5 min at room temperature with a streptavidin/peroxidase performed complex (Vector). Slides were washed twice as before and the solution of the chromogen substrate (diaminobenzidine) added for 5–10 min. After a 5-min wash in distilled water, slides were counterstained by 10 min of incubation with 0.2% ethyl green in 0.1 M sodium acetate buffer (pH 4.0). Finally, slides were rinsed in distilled water and, after 2 × 1.5 min dips in butanol and 3 × 2 min dips in xylene, mounted using the Eukitt mounting medium (Kindler O., GmbH & Co., Freiburg, Germany). DAKO ER and PR control slides were processed simultaneously and used as positive controls. Meanly, 100 randomly selected fields from at least three different slides were analyzed. Analysis of the receptor staining was performed using the quantitative estrogen/progesterone analysis application (release 2.0) for CAS 200 Image Analyzer (Becton-Dickinson Italia spa, Milan, Italy), which automatically yields the percent of positively stained nuclei and measures the intensity of staining; the latter was defined as the summed absorbance for the positive receptor nuclear area over the summed total absorbance of all of the nuclei expressed as a percentage. Specimens with ≥10% malignant cells stained were considered ER or PR positive.

Analysis of Intratissue Estrogens. Experimental procedures for tissue extraction, and subsequent HPLC and GC/MS of estrogens have been optimized previously and validated (24, 25), and are used currently in our laboratories for the analysis of urinary estrogens. Briefly, breast tissues were dissected, homogenized in a solution consisting of 3 ml 0.01 N $HClO_4$ and 3 ml 0.1 M Tris buffer (pH 8.6) at a final pH of 3.4, and centrifuged at 2000 × g for 10 min. The resulting supernatant was decanted into a test tube containing 40 mg of acid alumina in 5 ml of 0.1 M Tris buffer. The CCE adsorbed by acid alumina (pH 4.0) were extracted using 200 μl of 0.2 M acetic acid and dried in a Speed Vac system (Savant Instruments Inc., Farmingdale, NY), whereas all of the other estrogens were extracted from Tris buffer with diethyl ether and dried at 40°C. Both extracts were combined immediately before HPLC analysis. RP-HPLC analysis was carried out using a Beckman (Beckman Instruments Inc., Berkeley, CA) HPLC system equipped with a Spherisorb 5S ODS-2 (Phase SEP, Clwyd, United Kingdom) column, a model 160 UV detector, and an electrochemical detector Coulochem 5100A (ESA, Bedford, MA). Dried extracts were redissolved in 100 μl of acetonitrile and aliquots (10–20 μl) used for

RP-HPLC analysis, using computer-aided, optimized isocratic conditions, as reported previously (26, 27). To verify identity of individual metabolites resolved by RP-HPLC, GC/MS analysis was performed on single peak fractions collected from a chromatographic system after UV detection. GC/MS was carried out using a GC-MS Model 5890–5970 (HP, Rockville, MD), equipped with a 5% phenyl-methyl silicone capillary column (HP 190915–105). Dried extracts of individually collected RP-HPLC fractions were redissolved in 50 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide/*N*-trimethylsilylimidazole (1000:2, v/v; Pierce, Luton, United Kingdom) containing 0.2% dithioerythritol (Serva, Heidelberg, Germany), according to methods established previously for anabolic steroids and their hydroxylated derivatives (28). Equilin (50 ng) was added as an internal standard to dried extracts immediately before GC/MS analysis. Samples (1–2 μl) were injected with a splitter in the ratio 1:10. Helium was used as carrier gas at a flow rate of 0.9 ml/min. The energy of ionizing electron beam was 70 eV, and electron impact mass spectra were recorded in the full scan mode from 100 to 600 a.m.u. GC/MS analysis of single peaks was performed on a single ion of either internal standard Equilin or the specific external standard (20 ppm); calculation was achieved through area measurement integration, using an MSD 5890 software package. Data were also corrected for recovery values (range of 82–92%).

Statistical Analysis. The finding that mean and median age of breast cancer patients at diagnosis were identical (52 years) indicates that distribution is symmetrical (41 and 62 lowest and highest quartiles). Because of the non-normal distribution of values, all of the directly measured concentrations of intratissue estrogens were logarithmically transformed; this modification strongly normalizes asymmetrical data distribution and allows the use of parametric tests. To obtain a robust estimate of mean values regardless of side lengths we also computed the α-trimmed means ($\alpha = 0.5$). The degree of correlation among individual metabolites was computed using the Pearson correlation coefficient. Statistical analysis of differences in intratissue estrogen concentrations between normal and cancer samples, and in relation to tumor volume, lymph node involvement, tumor grading, and ER status, on one hand, and patient survival, on the other, was conducted through the parametric test of ANOVA and the Mann-Whitney nonparametric test. The impact of potential confounding variables (including age at diagnosis, menopausal status, tumor volume, lymph node involvement, tumor grading, ER status, and medical treatment) was assessed using unconditional logistic regression (29). Odds ratios and their 95% confidence intervals for individual variables were calculated and *P*s computed using Mantel's test for trend (see Table 1). Wherever appropriate, +/+ and −/+ tumors were combined and designated ER-positive for statistical comparisons with ER-negative (−/−) tumors. All of the *P*s resulted from two-sided statistical tests; $P\text{s} < 0.05$ were considered significant.

RESULTS

LBA of ERs. Distribution of different sites having higher (type I) and reduced (type II) affinity of estrogen binding was inspected in both soluble and nuclear fractions of breast tumor tissues. As indicated by our previous studies on breast and endometrial cancer patients, concurrent presence of type I ER in either cell fraction would identify a functional receptor status and be associated with a more favorable prognosis (30–32). Overall, 57.2% of patients had a homogeneously positive (+/+) or heterogeneous (−/+) type I ER status, with the remaining (42.8%) having homogeneously ER-negative (−/−) tumors. The dissociation constant values were below cutoff limit of 0.55 nM (range, 0.36–0.51 nM), indicating that type I (high affinity, limited capacity) ERs are commonly detected in soluble and/or nuclear compartment. ER concentrations were in the range of those we have observed previously in human breast tumors (19). Type II ERs were detected in a higher proportion of cases (ranging from 62 to 74%), although their presence was mostly concurrent with that of type I ER (not shown).

No statistically significant difference was observed in both DFS and OS of patients having ER +/+, −/+, or −/− tumors when either individually considered ($P = 0.811$) or after combining ER +/+ and −/+ cases (overall designated as ER-positive) compared with ER −/− ones ($P = 0.540$).

Immunohistochemical Studies. Quantitative estimation of ICAs of both ER and PR, using the CAS 200 Image Analysis System, yielded results equivalent to those obtained using LBA. The percentage of receptor-positive tumors was 50.6% for ER and 56.3% for PR. This picture corresponds to the proportion of tumors having ER-positive status in the nuclear fraction (57.2%). In the majority of cases, ER-positive tumor tissues stained positively also for PR, the percentage of positive nuclei ranging from 56.7 to 66.4% and from 59.0 to 72.6%, respectively, for ER and PR. The intensity of staining was consistently strong (ranges of 65.4–88.3% for ER and 62.8–84.9% for PR), with a coefficient of variation of 15–18%. Overall, a concordance of 65% for ER- or PR-positive cases with ER +/+ tumors was observed. Notably, >88% of ER-positive cases had also nuclear ER by LBA. In addition, a significant ($P < 0.02$) correlation was found comparing ER levels, measured as percentage of positive area (≥10% cutoff value), with nuclear ER concentrations by LBA, expressed as fmol/mg DNA (not shown).

Intratumor Estrogen Content. Separation, identification, and estimation of individual estrogens was conducted using RP-HPLC and GC/MS approaches. In particular, the GC/MS confirmed the identity of all of the peak fractions collected from RP-HPLC after UV detection. A pointed example of selected ion chromatogram of a tissue extract with prior HPLC purification is illustrated in Fig. 1.

As also observed previously (25), the use of both acid alumina extraction and RP-HPLC purification markedly enhanced recovery values and signal:background ratio for MS analysis, yielding consistent results for quantitation of individual metabolites.

As reported in Table 2, variable concentrations of several hydroxylated estrogens, including 16αOHE_1, 2 and 4OHE_1, 2 and 4OHE_2, and their respective MeO derivatives (2 and 4$MeOE_1$, and 2 and 4$MeOE_2$), were detected, and this was especially true in normal tissue samples. It is worth noting that hydroxylated and MeO estrogens accounted for nearly 95% of the total, with classical estrogens representing only 5% and 4% of all of the estrogens in normal and cancer cases, respectively.

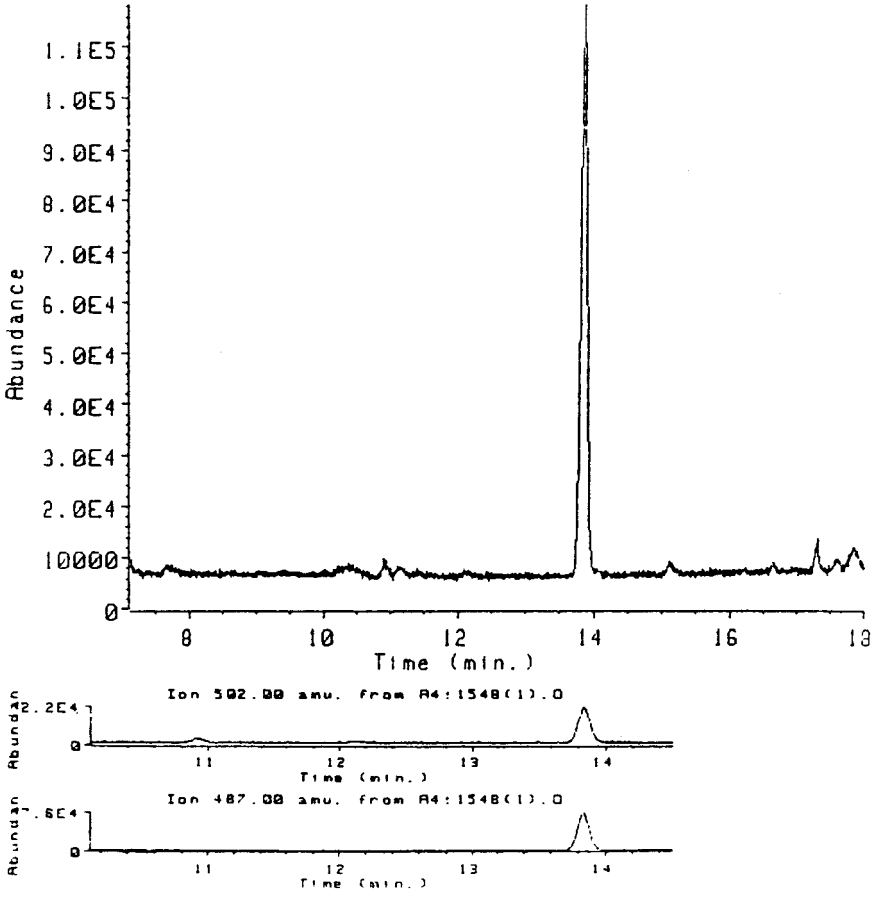

Fig. 1 Selected ion gas chromatogram of the purified peak fraction from a breast cancer tissue extract. Ions monitored at *m/z* 502 (M^+) and 487 (M-15) for 16αOHE_1.

A trend for larger (T_3-T_4) tumors to exhibit greater total amounts of hydroxyestrogens was also observed (not shown).

Overall, intratumor estrogen amounts were nearly 2-fold greater than those observed in normal tissues; in particular, both 2- and 4-hydroxyestrogens were consistently elevated in cancer, with 2- and $4OHE_2$ being significantly increased ($P = 0.02$ and $P = 0.001$, respectively) with respect to normal tissues. By contrast, neither 2- nor 4-MeO derivatives exhibited substantial changes, except for a significant ($P = 0.024$) rise of $2MeOE_1$ in cancer cases. Interestingly, the average 16α-hydroxylation showed a nearly 4-fold, although not significant, increase in tumor tissues, but it remained consistently lower (7-fold and 9-fold, respectively) than 2- and 4-hydroxylation (see Table 2). However, the 2:16αOHE_1 ratio values were significantly ($P = 0.024$) higher in cancer than in normal samples. In tumor tissues, the $4OHE_2$ was the most prevalent metabolite, its concentration being more than three times greater than that of $4OHE_1$, whereas intermediate, equivalent amounts of $2OHE_2$ and $2OHE_1$ were seen. Conversely, normal tissues revealed a marked prevalence of 2- and, especially, 4-OHE_1 over hydroxylated derivatives of E_1. Overall, MeO estrogens accounted for a limited part (range of 7–14%) of all estrogens in both normal and cancer tissues (see Table 2).

No significant association was found between intratumor levels of any measurable hydroxyestrogen and tumor volume,

Table 2 Intratissue concentrations[a] of estrogens, hydroxyestrogens, and their methoxy derivatives in normal and malignant breast

Estrogen[b]	Normal ($n = 6$)	Cancer ($n = 17$)	P^c
E_2	20.3 ± 8.6	41.4 ± 11.3	0.431
E_1	19.5 ± 5.1	28.9 ± 6.5	0.562
E_3	105.7 ± 49.1	121.3 ± 39.3	0.919
16αOHE_1	61.5 ± 26.3	232.2 ± 54.6	*0.074*
2OH-	887.3 ± 588.2	1568.8 ± 336.7	*0.062*
-E_1	741.7 ± 610.8	785.1 ± 160.3	0.101
-E_2	145.7 ± 95.6	790.5 ± 311.3	**0.020**
2:16αOHE_1	8.3 ± 4.6	1.5 ± 0.11	**0.024**
4OH-	1302.0 ± 821.2	2098.9 ± 437.5	*0.093*
-E_1	1223.3 ± 836.8	492.5 ± 80.1	0.609
-E_2	78.7 ± 49.5	1608.7 ± 466.8	**0.001**
2MeO-	154.2 ± 91.4	158.1 ± 26.8	0.363
-E_1	21.7 ± 11.7	90.4 ± 20.6	**0.024**
-E_2	132.5 ± 93.7	67.7 ± 18.7	0.609
4MeO-	262.2 ± 193.6	224.6 ± 58.9	0.207
-E_1	130.5 ± 97.6	49.1 ± 15.7	0.919
-E_2	131.7 ± 94.7	175.5 ± 50.6	0.101
Total	2812.7 ± 1283.3	4655.3 ± 760.1	

[a] Means ± SEs of the means (fmol/mg tissue).

[b] 2OH- = $2OHE_1$ + $2OHE_2$; 4OH- = $4OHE_1$ + $4OHE_2$; MeO- = $MeOE_1$ + $MeOE_2$; $MeOE_1$ = 2- and 4-methoxyestrone; $MeOE_2$ = 2- and 4-methoxyestradiol.

[c] Mann-Whitney test (see "Statistical Analysis" section for details). Comparisons where significant differences are found are marked in bold, those showing a trend towards significance are marked in italic.

Table 3 Distribution of intratissue hydroxyestrogens according to the ER status of breast tumors[a]

ER status[b]	Hydroxyestrogens			
	$16\alpha OHE_1$	$2OHE_1$	$4OHE_1$	$2OHE_1$:$16\alpha OHE_1$
Positive (n = 12)	2.285 (1.961–2.609)	2.673 (2.346–3.001)	2.773 (2.596–2.949)	1.25 (0.983–1.511)
Negative (n = 9)	1.823 (1.423–2.224)	2.856 (2.653–3.058)	2.575 (2.256–2.895)	1.65 (0.324–2.916)
	P = 0.058	P = 0.366	P = 0.210	P = 0.048
	$2OHE_2$	$4OHE_2$	$4OHE_2$:$2OHE_2$	
Positive (n = 12)	2.530 (2.088–2.972)	3.027 (2.587–3.467)	5.10 (1.005–9.199)	
Negative (n = 9)	2.234 (1.753–2.716)	2.314 (1.704–2.924)	3.42 (0.151–6.686)	
	P = 0.333	P = 0.100	P = 0.380	

[a] Means and (95% confidence intervals) of logarithmically transformed data.
[b] ER-positive group aggregates ER +/+ and −/+ tumors; ER-negative group includes ER −/− tumors only.

grading, or nodal status, except a trend (P = 0.072) for $2OHE_1$ to have greater concentrations in patients without lymph node involvement (not shown). As far as the ER status is concerned (see Table 3), remarkably higher (although not significant: P = 0.058) $16\alpha OHE_1$ concentrations were observed in ER-positive tumors, whereas neither $2OHE_1$ nor $4OHE_1$ showed any correlation with the receptor status. However, $2OHE_1$:$16\alpha OHE_1$ ratio values were significantly (P = 0.048) lower in ER-positive tumors. In particular, a strong inverse relationship was found between $2OHE_1$:$16\alpha OHE_1$ ratio values and $16\alpha OHE_1$ but not $2OHE_1$ concentrations, suggesting that this association is dominated by $16\alpha OHE_1$ (Fig. 2). Conversely, $4OHE_2$ showed greater concentrations in ER-positive tumors (P = 0.100), whereas $4OHE_2$:$2OHE_2$ ratio values did not correlate with ER status (P = 0.380).

Although no significant association was found between any hydroxyestrogen and DFS, statistically significant differences were observed for individual metabolites and/or their ratios in relation to the OS of patients. As shown in Fig. 3, significantly greater (P = 0.015) amounts of $16\alpha OHE_1$ were in fact seen in patients surviving with respect to patients who died of breast cancer (mean values respectively of 284.0 and 71.1 fmol/mg tissue); by contrast, $2OHE_1$ showed equivalent concentrations in the two patients groups ($\bar{x}$ = 890.6 and 776.4 fmol/mg tissue, respectively). However, $2OHE_1$:$16\alpha OHE_1$ ratio values were significantly lower (P = 0.043) in patients alive (Fig. 4), supporting the concept that this association is mostly governed by $16\alpha OHE_1$, exactly as it is seen for the correlation between this ratio and the ER status of tumors.

Regarding 2- and 4-OH-E_2 derivatives, 1.6-fold higher levels of $4OHE_2$ were observed in surviving patients, whereas $2OHE_2$ concentrations were 3-fold greater in women who died from disease. Interestingly, the ratio of 4:2OH+MeO derivatives of both E_2 and E_1 showed significantly lower (P = 0.006) values in deceased breast cancer patients (0.712) than in their surviving counterparts (3.023). In an attempt to verify whether combination of different hydroxyestrogens or their ratios may results in an improved ability of discriminating patients having

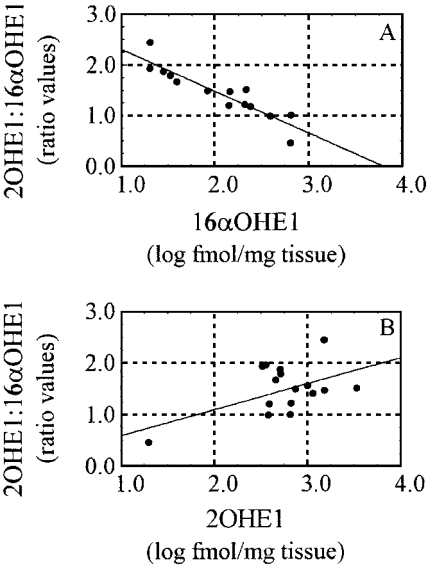

Fig. 2 Correlation of 2:$16\alpha OHE_1$ ratio values and intratumor concentrations of (*A*) $16\alpha OHE_1$ and (*B*) $2OHE_1$. Pearson correlation coefficients: A = 0.845 (P < 0.01); B = 0.231 (P = 0.371).

distinct survival probability, we have related the 4:2OH+MeO ratio values to $16\alpha OHE_1$ concentrations in both alive and dead patients. As illustrated in Fig. 5, using arbitrary cutoff values of 1.2 for 4:2OH+MeO ratio and 150 fmol/mg tissue for $16\alpha OHE_1$, we were able to achieve a clear-cut separation of the two groups of patients. In particular, over-cutoff patients had a median survival time of 147 months, whereas under cutoff patients showed only 47 months survival (P = 0.00008).

DISCUSSION

In the present work we have measured intratissue levels of main estrogens, hydroxyestrogens, and their MeO derivatives in breast tumors, in relation to follow-up of patients. The resulting

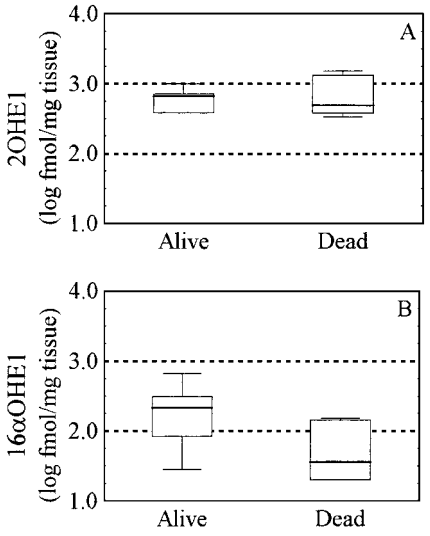

Fig. 3 Intratumor concentrations of (*A*) $16\alpha OHE_1$ and (*B*) $2OHE_1$ in relation to patients survival. Median values, lowest and highest quartiles, minimum and maximum values are shown. Median values and interquartile ranges were: *A*, alive, 2.33 and 0.78; dead, 1.56 and 0.86; $P = 0.015$; *B*, alive, 2.82 and 0.29; dead, 2.69 and 0.58; $P = 0.815$; *bars*, ±SD.

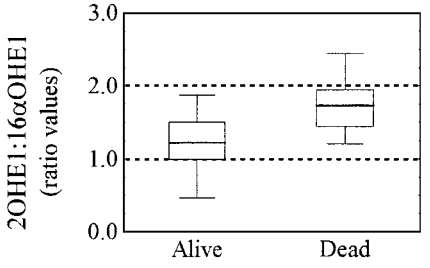

Fig. 4 Values of $2{:}16\alpha OHE_1$ ratio in relation to patients survival. Median values, lowest and highest quartiles, minimum and maximum values are shown. Median values and interquartile ranges were: alive, 1.22 and 0.54; dead, 1.73 and 0.53; $P = 0.043$; *bars*, ±SD.

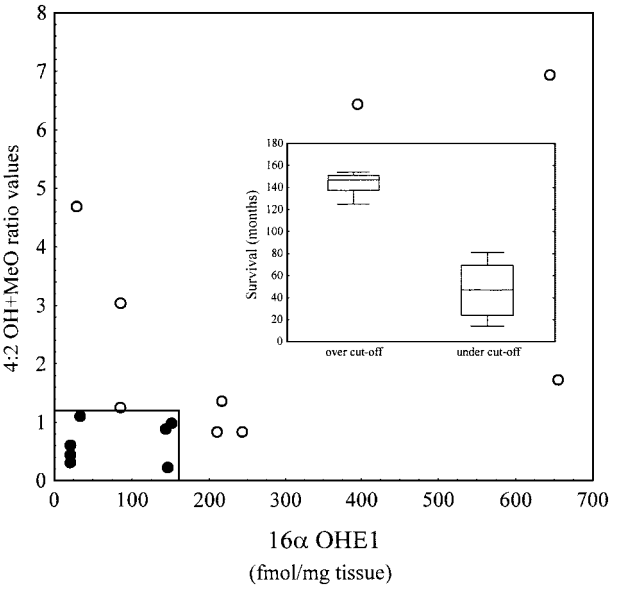

Fig. 5 Values of 4:2OH+MeO E_2 and E_1 ratio, and intratumor concentrations of $16\alpha OHE_1$ in relation to patients survival. ○, alive; ●, dead. *Inset*, patients having shorter (47 months) and longer (147 months) median survival times were separated using arbitrary cutoff values of 1.2 for 4:2OH+MeO ratio and 150 fmol/mg tissue for $16\alpha OHE_1$ ($P = 0.00008$); *bars*, ±SD.

data were also compared with a set of histologically normal mammary tissues used as control.

We report that intratumor levels of $16\alpha OHE_1$ are significantly higher in patients having longer survival time after >12 years of mean follow-up. Conversely, $2OHE_1$ did not show any remarkable variation in relation to patient survival. However, when considering $2OHE_1{:}16\alpha OHE_1$ and 4:2OH+MeO ratio values, both showed different patterns, being significantly lower and greater in surviving patients, respectively. This association was independent of other potentially confounding variables including age and menopausal status of patients, tumor volume, lymph node involvement, tumor grading, and ER status, as well as medical treatment.

No statistically significant difference was observed in either DFS or OS of patients having ER +/+, −/+, or −/− tumors ($P > 0.8$), although the limited number of cases does not allow us to draw any conclusive inference. However, we have reported previously that, in larger breast cancer studies, patients whose tumors have a homogeneously positive (+/+) ER status show a significant survival advantage with respect to patients having homogeneously ER negative (−/−) tumors; no significant difference could be observed in patients with heterogeneous (−/+) ER status and those having +/+ or −/− tumors (31, 32). It should be pointed out that the ligand binding and ICAs we have used to measure ER in breast tumor tissues almost exclusively identify the classical ERα, as corroborated by the high-affinity characteristics of estrogen binding and by the use of specific antibodies that do not cross-react with ERβ, respectively. In this respect, the expression of ERβ in breast cancer and its potential association to either patient prognosis and intratissue estrogens remains to be determined.

The assumption that specific hydroxyestrogens and/or their reactive intermediates may have important biological activities, favoring neoplastic transformation of mammary epithelial cells or affecting the proliferative activity of breast tumor cells, has been repeatedly reported and debated. On the basis of the concept that C-2 and C-16α hydroxylations represent mutually exclusive pathways of estrogen metabolism and that the resulting hydroxylated metabolites may respectively behave as estrogen antagonists and agonists (33), much attention has been devoted in the last 2 decades to define the potential role of an imbalance between 2- and 16α-hydroxylation activities in relation to breast cancer. Previous studies have reported that the extent of 16α-hydroxylation is significantly elevated in mice strains at high incidence of spontaneous mammary tumors, as well as in breast cancer patients compared with matched healthy controls or in women at high risk of developing breast cancer (34–37). On the other hand, Bradlow *et al.* (38) revealed that, using various experimental models, any increase in 2-hydroxylation

eventually led to protection against tumor development and *vice versa*; on this basis, the authors developed the concept of $2OHE_1$ as the good estrogen, having anticarcinogenic activity.

In our study, greater intratumor amounts of $16\alpha OHE_1$ and lower $2OHE_1$:$16\alpha OHE_1$ ratio values are significantly associated with a prolonged survival of breast cancer patients. Although this evidence is apparently in conflict with the implication of $16\alpha OHE_1$ as either initiator or promoter of human mammary carcinoma, the potential role of this metabolite, as well as that of other hydroxyestrogens, in the onset and the clinical progression of breast cancer should be kept separated. We have in fact revealed that normal breast tissues have markedly lower amounts of $16\alpha OHE_1$ when compared with cancer tissues, although this difference shows only a trend ($P = 0.074$) toward significance. However, significantly lower values of 2:$16\alpha OHE_1$ ratio were observed in tumor tissues, reinforcing the hypothesis that an imbalance of 2- and 16α-hydroxylation may be implicated in breast cancer development. On the other hand, intratumor levels of $4OHE_2$ were significantly greater than those seen in normal breast tissues, supporting previous reports by which metabolic conversion of E_2 to $4OHE_2$ and its additional activation to reactive semiquinone/quinone intermediates may have an important role in the carcinogenetic process (1, 8, 39).

Previous *in vivo* studies have reported a strong positive association of $16\alpha OHE_1$ with breast cancer by scrutiny of women at high familial risk or comparing breast cancer cases to healthy controls (40, 41). However, these studies have repeatedly been questioned because neither methodologies nor results could be validated by other authors (42, 43). Ursin *et al.* (44), in a recent study on urinary $2OHE_1$ and $16\alpha OHE_1$, were unable to support the hypothesis that the ratio of 2:$16\alpha OHE_1$ is a risk factor for breast cancer, although some issues in their study have also been argued (45). It ought to be emphasized here that all of the previous studies have merely related the extent of both 2- and 16α-hydroxylation of E_2, as like as urinary levels of the resulting products, to breast cancer risk, whereas the potential role of individual hydroxyestrogens in the progression of the disease has never been addressed.

On the basis of all of this inconsistency, Liehr (1) has claimed the need for the assessment of tissue content of hydroxyestrogens, because specific and local formation of individual metabolites and the resulting balance may lead to their differential accumulation and differently affect both tumor development and progression. To our knowledge, this is the first report by which local formation and metabolic profiles of estrogens have been measured in both normal and tumor breast tissues and related to the severity of disease.

Some previous studies have measured intratissue amounts of classical estrogens (mostly E_2 and E_1) in normal, benign, or malignant breast from both premenopausal and postmenopausal women, also in relation to their respective plasma values. Although the different methods and procedures used often resulted in a large discrepancy of data, there is an overall consistency among different reports, in particular: (*a*) intratissue levels of estrogens are significantly higher than respective plasma values; (*b*) markedly greater concentrations of E_2 are found in cancer with respect to either normal or benign breast tissues, irrespective of menopausal status; (*c*) no significant correlation between ER content and intratissue levels of E_2 is observed in breast cancer patients (15, 17, 46, 47). Whereas our data mostly conform with this evidence, both the steroid extraction procedures and the RP-HPLC/GC-MS analysis we used to assess intratissue content of estrogens have permitted the concurrent inspection of classical, OH, and MeO estrogens, and allowed us to compare levels of individual estrogens in normal and malignant mammary tissues, and to relate them to follow-up of breast cancer patients.

The present study, although hindered by the limited number of breast cancer cases, clearly reveals that elevated intratumor concentrations of $16\alpha OHE_1$ associate with a prolonged survival of patients. In addition, by combining arbitrary cutoff values for both $16\alpha OHE_1$ and 4:2OH+MeO ratio, we were able to achieve a distinct separation of patients having significantly different survival times. In this respect, lower values of both $16\alpha OHE_1$ and ratio of 4- to 2-hydroxyestrogens apparently identify patients having an unfavorable prognosis; conversely, neither 2- nor 4-OHE_1 did correlate to patient OS. The trend we observed for larger tumors having greater hydroxyestrogen contents, along with the reported significant association of individual metabolites and clinical progression of breast cancer, strongly suggest that these estrogen derivatives represent direct products of cellular metabolism rather than being the result of a differential uptake from circulation. This concept is also supported by evidence from our previous *in vitro* studies on estrogen metabolism in human breast tumor cells (18, 48).

It is likely that changes in specific cytochrome P450 steroid enzymes are responsible for high local estrogen formation and metabolism in breast tumor tissues, independent of the uptake from circulation. There is emerging evidence that high levels of aromatase (CYP19) expression and/or activity in breast cancer could result in elevated intratumor estrogens, either E_2 or E_1 (49, 50). In addition, both the E_2-4-hydroxylase (CYP1B1) and the 16α-hydroxylase P450 enzymes have been, in turn, implicated in human mammary carcinogenesis (51, 52). In the present work, we report greater amounts of 2- and 4-OHE_2, as well as of $16\alpha OHE_1$ in tumor as compared with normal breast tissues. However, it remains to be clarified whether the correlation we have observed between elevated intratumor concentrations of $16\alpha OHE_1$ or $4OHE_2$ and ER-positive breast cancer or prolonged survival of patients represent tumor-associated changes or could rather be regarded as biologically important to explain the ostensible effects of these metabolites on the progression of disease. In this latter case, the prolonged time of nuclear residence and/or the formation of highly reactive intermediates of specific hydroxyestrogens may be considered as essential prerequisite to conceive their potential biological impact. In this respect, one could speculate that individual estrogen derivatives may locally behave as antiestrogens by competing with E_2 for either receptor occupation or interaction with hormone response element of estrogen-sensitive genes, or both.

Anyhow, the present results strongly suggest the reconsideration of the potential implication of hydroxyestrogens in the onset of human breast cancer as distinct from their role in the progression of disease.

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