

Antiestrogenic effects of the fetal estrogen estetrol in women with estrogen-receptor positive early breast cancer

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Estetrol (E4) is a fetal estrogen with estrogenic effects on reproductive organs and bone in preclinical models and in postmenopausal women. However, E4 exerts antiestrogenic effects on breast cancer (BC) cell growth *in vitro* and *in vivo*. We have investigated the effect of 14 days preoperative treatment with 20 mg E4 per day on tumor proliferation markers, sex steroid receptor expression and endocrine parameters in a prospective, randomized, placebo-controlled, preoperative window trial in 30 pre- and postmenopausal women with estrogen-receptor positive early BC. E4 had a significant pro-apoptotic effect on tumor tissue, whereas Ki67 expression remained unchanged in both pre- and postmenopausal women. E4 increased sex-hormone-binding globulin significantly thereby reducing the concentrations of bioavailable estradiol. Follicle-stimulating hormone levels decreased in postmenopausal women only and luteinizing hormone levels remained unchanged. Systemic insulin growth factor-1 levels decreased significantly. Intratumoral epithelial ER α expression decreased significantly and a trend was found towards an increased expression of ER β . This clinical data support the preclinical findings that E4 has antiestrogenic effects on BC cells, whereas earlier studies have shown that E4 has estrogenic effects on reproductive tissues and bone. Further clinical studies seem acceptable and are needed to confirm the safety and efficacy of E4 for the breast in hormone replacement therapy, including hormone replacement therapy in women who have or have had BC, especially in those BC patients treated with aromatase inhibitors and suffering from serious complaints due to estrogen deficiency.

Introduction

The natural estrogen estetrol (E4) is exclusively synthesized by the human fetal liver during pregnancy. E4 was discovered in 1965 at the Karolinska Institute in Stockholm (1) and differs from estradiol (E2) by an additional α -hydroxy (OH) group at position 15 of the molecule. This single additional OH group (E3) extends the elimination half-life after oral administration in the human from 10 to 20 min for E3 to 28 h for E4 (2). This property together with its high bioavailability makes E4 suitable as an oral drug.

E4 binds to both estrogen receptor (ER)- α as well as ER- β with a low affinity compared with ethinylestradiol and estradiol (E2) and does not bind to other steroid receptors and to a panel of 130 other drug targets (3). Metabolism of E4 in human liver cells is slow and no active metabolites have been detected (3). Contrary to ethinylestradiol and especially E2, E4 does not bind to sex-hormone-binding globulin

(SHBG) (4) and does not inhibit the activity of the most important cytochrome P-450 liver enzymes (3). E4 is excreted in an inactive form by the liver and the kidney following conjugation to sulfate and/or glucuronide (5). Pharmacological studies have demonstrated that E4 acts as an estrogen on the vagina (6), uterus (6) and bone (7). E4 suppresses hot flush-like temperature rises in an animal model (8), inhibits ovulation in the rat (9) and has a vasorelaxing effect on isolated rat arteries (10). In animal studies with doses of up to 10 mg/kg/day for 4 weeks, E4 appeared to be safe and did not cause relevant side effects.

In the human, a daily oral dose of 2 mg E4 had an estrogenic effect on vaginal cytology, comparable with 2 mg E2-valerate. Median endometrial thickness did not increase with 2 mg E4, whereas with E2-valerate and 10 mg E4, endometrial proliferation was observed. With E4, follicle-stimulating hormone (FSH) levels decreased and SHBG levels increased dose dependently. Biochemical bone formation and resorption parameters demonstrated a significant and dose-dependent decrease of bone turnover. Lipids showed dose-dependent estrogenic changes and a stronger increase of triglycerides with E2-valerate. More details are available in review papers (11,12)

Rather surprisingly, during the pharmacological profiling of E4 in human breast cancer (BC) cell lines and in the *in vivo* 7,12-dimethylbenz(a)anthracene rat breast tumor model, E4 was found to exhibit estrogen antagonistic effects, especially but not exclusively in the presence of E2. In three separate experiments in the 7,12-dimethylbenz(a)anthracene model, E4 dose dependently prevented the occurrence of breast tumors and caused regression of existing tumors (13). High doses of E4 up to 10 mg/kg/day were as effective as ovariectomy in removing tumors and caused no side effects during 4 weeks of treatment.

To study the potential antitumor effect of E4 in human BC, a prospective, randomized, double-blind, placebo-controlled, preoperative window study was performed, comparing 14 days preoperative administration of a daily dose of 20 mg E4 with placebo treatment in 15 pre- and 15 postmenopausal women with newly diagnosed ER-positive early BC. This is the first clinical study performed with E4 in women with BC.

Materials and methods

This prospective randomized clinical investigation (ClinicalTrials.gov Identifier: NCT00464516) complied with the Declaration of Helsinki and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guideline for Good Clinical Practice (CPMP/ICH/135/95). The study was approved by the institutional review board of the Medical University of Vienna and the Austrian Competent Authorities AGES. The trial was monitored regularly. All patients had provided written informed consent prior to study enrolment. The aim of this study was to evaluate the effects of E4 on intratumoral apoptosis and apoptosis-related proteins, on the expression of the proliferation marker Ki67, on sex steroid hormone receptor expression, on serum hormone levels and on safety and tolerability.

Patient characteristics

Eligible patients had to have histologically confirmed ER-positive early-stage BC and had to be scheduled for curative surgery. Body mass index had to be between 18 and 32 kg/m². Distant disease had to be ruled out by chest x-ray, liver sonography and bone scan prior to enrolment. Both pre- and postmenopausal women were eligible, but the menopausal status had to be known prior to randomization. Patients were randomized 2:1 to receive either 20 mg of E4 or placebo once daily for 14 \pm 2 days. Concomitant treatment with systemic chemo-, endocrine- or radiotherapy was not permitted.

Assessments

BC tissue was obtained by core biopsies prior to study treatment and during BC surgery at the end of the treatment period. Expression of tumor cell proliferation-associated Ki67, estrogen receptor α (ER α), estrogen receptor β (ER β), progesterone receptor, Bax, Bcl2 and insulin growth factor-1 (IGF-1)

Abbreviations: BC, breast cancer; E2, estradiol; E4, estetrol; ER, estrogen receptor; FSH, follicle-stimulating hormone; IGF-1, insulin growth factor-1; SHBG, sex-hormone-binding globulin.

receptor was analyzed by immunohistochemical analysis in formalin-fixed, paraffin-embedded tissue, and apoptosis was evaluated by using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method (14). ER α , ER β and progesterone receptor protein expression was evaluated by using the Remmele score (15). Serum was obtained before (day 0), during (days 7 and 14) and 7 days after (day 21) the treatment phase for analysis of FSH, LH, E2, bioavailable E2, progesterone, testosterone, bioavailable testosterone, androstenedione, SHBG, prolactin and IGF-1.

Safety and tolerability were assessed by monitoring adverse events, physical examination and laboratory evaluation (blood biochemistry and hematology). Endometrial thickness was measured by transvaginal ultrasound and endometrial tissue was obtained by curettage during the surgical procedure and histological examination was performed.

Statistics

Statistical analyses were performed using SPSS 15.0.1 (SPSS Benelux, Gorinchem, The Netherlands). Variables were checked for Gaussian distribution with the Shapiro-Wilks test. Levene's test for equality of variance was used to estimate the probability that treatment groups had different variances. Effects of experimental treatment (between-subject factor: E4 and placebo) and menopause (between-subject factor: pre- and post-menopausal) on study parameters were analyzed using a repeated-measures analysis of variance with time (D0 and D14) as within-subject factor. *Post hoc* analyses were performed on significant treatment. Univariate analysis of variance was performed on all parameters after 14 days of treatment (D14) with treatment (E4 and placebo) and menopause (premenopausal and postmenopausal) as between-subject factors. Data are presented as mean \pm standard error of the mean unless otherwise indicated. Differences were considered significant at $P < 0.05$ in two-tailed analyses.

Results

Patient characteristics

Of the 38 BC patients who were screened, 8 patients were excluded because of withdrawal of informed consent ($n = 5$), inappropriate body mass index ($n = 2$) and use of hormonal contraceptives ($n = 1$). The remaining 15 pre- and 15 post-menopausal women were included in the trial and randomized 2:1 to receive either 20 mg E4 once daily or placebo. One post-menopausal patient, randomized into the placebo arm, was lost for follow-up after randomization, so 15 pre- and 14 post-menopausal patients were eligible for response evaluation as intention-to-treat population. Both treatment groups were well balanced for known clinico-pathological factors (Table I).

Effect of E4 on intratumoral Ki67 and apoptosis index

Ki67 protein expression was evaluated by immunohistochemistry and pretreatment samples were available for all 30 randomized patients. However, because of pathological complete response and inadequate tissue sampling or analysis matched pre- and post-treatment samples were only available for 10 patients who had received E4 and for 3 patients who had received placebo. The 14 day treatment with E4 as well as with placebo did not significantly change the expression of Ki67 in tumor tissue, but ranges were large (Figure 1). E4 treatment did, however, cause a significant increase in the number of apoptotic cells when compared with placebo treatment (Figure 2, $P < 0.05$). The increase in the apoptosis index was significant in both pre- and post-menopausal women ($P < 0.05$, data not shown). No significant changes were observed in the differential expression of the Bcl2 and Bax proteins (data not shown).

Effect of E4 on intratumoral sex steroid receptor expression

Using the Remmele scoring system, we evaluated the differential intratumoral ER α and ER β protein expression in matched sets from individual patients treated with either 20 mg E4 once daily or placebo for 14 days (Figure 3). E4 treatment caused a significant decrease in ER α expression ($P < 0.05$, Figure 3, left panel). A trend towards an increased level of expression of intratumoral ER β was observed (Figure 3, right panel). Figure 4 depicts representative photomicrographs of ER α (a and b) and ER β (c and d) immunostaining of tumor samples before (a and c) and after (b and d) 2 weeks of E4 treatment.

Table I. Clinico-pathological characteristics of patients participating in the study

	E4 ($n = 21$)	Placebo ($n = 8$)	P value
Mean age (range)	51.7 (37–68)	55.1 (40–70)	0.827
Menopausal status			
Premenopausal	11 (52%)	4 (50%)	1.000
Postmenopausal	10 (48%)	4 (50%)	
Histological subtype			
Ductal carcinoma	12 (57%)	8 (100%)	0.067
Lobular carcinoma	9 (43%)	0 (0%)	
Grading			
G1	4 (19%)	4 (50%)	0.238
G2	14 (67%)	3 (38%)	
G3	3 (14%)	1 (12%)	
Tumor stage			
T1	8 (38%)	5 (63%)	0.454
T2	12 (57%)	3 (37%)	
T3	1 (5%)	0 (0%)	
Nodal stage			
N0	19 (91%)	6 (75%)	0.552
N1	2 (9%)	2 (25%)	
ER status			
Positive	21 (100%)	8 (100%)	1.000
Negative	0 (0%)	0 (0%)	
PR status			
Positive	18 (86%)	7 (88%)	1.000
Negative	3 (14%)	1 (12%)	

Progesterone receptor expression was not affected by E4 treatment in both pre- and post-menopausal women (data not shown).

Effect of E4 treatment on endocrine parameters

E4 causes a significant increase in SHBG levels, an effect, which was observed in both pre- and post-menopausal women ($P < 0.05$, Figure 5a). E2 levels decreased in premenopausal women, although not significantly due to the large range of levels observed. Bioavailable E2 decreased also; in premenopausal women due to the decline of E2 and in E4-treated women due to the increased binding to the higher SHBG levels. E2 did not decrease in postmenopausal women treated with E4, whereas bioavailable E2 did decrease due to the rise of SHBG. E4 treatment resulted in a significant and pronounced decrease of FSH levels in postmenopausal women, which was evident already after 7 days and showed a further decrease after 14 days of treatment ($P < 0.05$, Figure 5b). In premenopausal women, E4 did not significantly alter FSH levels. Bioavailable testosterone decreased significantly ($P < 0.05$) in response to E4 in both the pre- and post-menopausal group (Figure 5c).

Serum IGF-1 levels decreased significantly with E4 both in pre- and post-menopausal women ($P < 0.05$, Figure 5d). However, E4 did not upregulate the intratumoral IGF type I receptor, measured by immunohistochemistry (data not shown).

E4 treatment resulted in an increase in prolactin levels in pre- and post-menopausal women when compared with placebo (Table II).

No significant changes were observed in the levels of LH, total testosterone and androstenedione (Table II).

Safety and tolerability

No serious side effects occurred related to E4 treatment. In total, 67 adverse events were reported between the start of study medication and completion of the study, but all were minor and/or short-lasting. Transvaginal ultrasound was performed at randomization and immediately before surgery to evaluate endometrial thickness. E4 treatment in post-menopausal women resulted in a trend towards an increased endometrial thickness ($P = 0.056$) and proliferation, whereas no change was observed during placebo treatment. Menstrual cycle changes in premenopausal women interfered with interpretation of endometrial thickness.

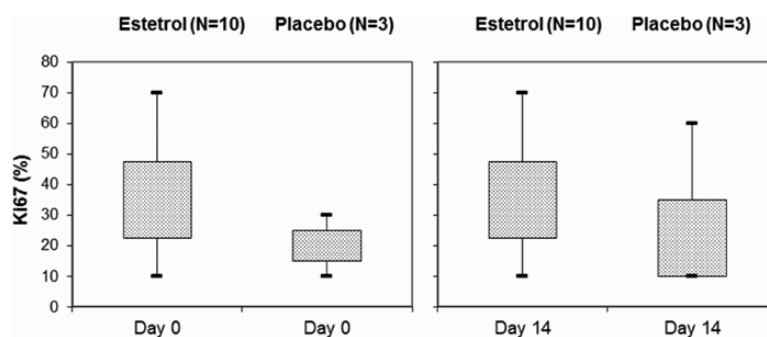


Fig. 1. Ki67 expression (%) in breast tumor tissue before (day 0) and after 14 days of oral treatment with 20 mg E4 or placebo per day.

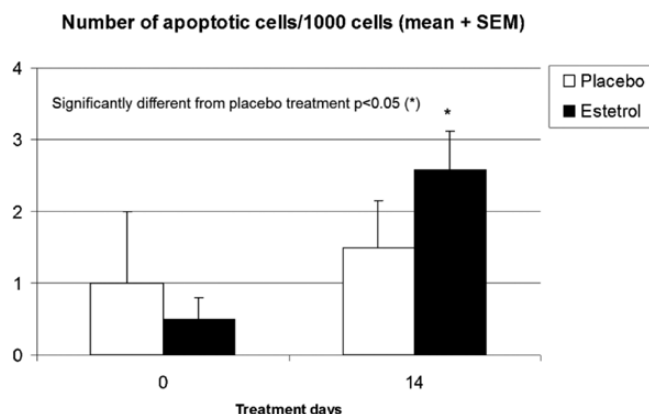


Fig. 2. Mean number of apoptotic cells in breast tumor tissue before (day 0) and after 14 days of oral treatment with 20 mg E4 (black bars) or placebo (white bars) per day.

Discussion

We here report the first study that investigates the effect of the natural human fetal estrogen E4 on BC in women. The most important findings in this small exploratory window trial in 29 women with early BC are that E4 significantly increases apoptosis, does not stimulate proliferation and induces downregulation of ER α and upregulation of ER β . This data confirm earlier observations from *in vitro* and *in vivo* studies, suggesting antitumoral effects of E4 on BC cells.

At present, aromatase inhibitors are considered to be the gold standard in the treatment of endocrine-sensitive BC in post-menopausal women, but their benefit is compromised by a number of side effects that are associated with a low estrogen environment, such as hot flushes, vaginal dryness, bone loss and musculoskeletal pain (16,17). These side effects can result in a reduced quality of life and consequently translate into poor drug compliance (18). In premenopausal women, aromatase inhibitors are contra-indicated since the suppression of estrogens elicits a positive central feedback resulting in an increase in FSH, which stimulates follicular development and, in consequence, ovarian E2 production. Tamoxifen with or without concomitant ovarian suppression is therefore still the standard therapy in these patients but particularly ovarian suppression by gonadotropin releasing hormone analogs in young women is very often compromised by severe menopausal symptoms including a decrease in bone density. In the light of its beneficial effect on menopausal symptoms and in consideration of the fact that in both pre- and post-menopausal patients E4 actually resulted in a decrease in FSH serum levels, it therefore appears that E4 could be a safe and effective add-back therapy, also in young symptomatic BC patients during antihormonal treatment.

Intratumoral Ki67 is an established prognostic parameter in BC, and changes in Ki67 in response to neoadjuvant endocrine therapy have been shown to be associated with disease-free outcome in a number of trials. Early changes in neoadjuvant Ki67 expression in response to endocrine therapy are surprisingly accurate in predicting the clinical outcome in the

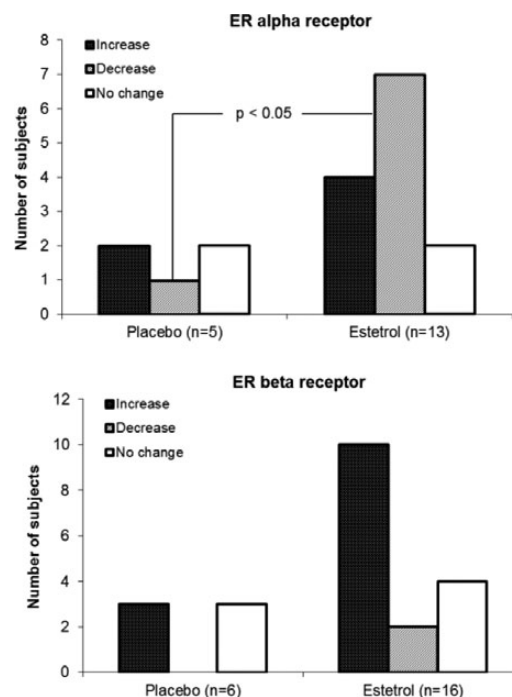


Fig. 3. Increase (dark gray bar), decrease (light gray bar) or no change (white bar) in intratumoral expression of ER α and ER β in response to 14 days of oral treatment with 20 mg E4 or placebo per day.

respective adjuvant trials (19). We have, therefore, evaluated Ki67 protein expression in both pre- and post-treatment samples from E4 and placebo-treated BC samples. No significant changes in response to E4 were found, but in view of the limited number of evaluable paired samples particularly in the placebo group ($n = 3$) and because of the potential of considerable intratumoral heterogeneity in Ki67 protein expression, the Ki67 data from this study must be interpreted with caution. Furthermore, several publications have recently shown considerable intra- and inter-observer variations, particularly in intermediate grade carcinomas, and when Ki67 expression was measured in accuracy steps of 5% and higher (20–22).

In addition, because the institutional review board mandated that in core-biopsy-proven BC the pretreatment study biopsy was only to be obtained after the patient had provided his/her informed consent, this required a second biopsy, and local inflammation and wound healing processes in response to the initial diagnostic biopsy might have impacted on Ki67 and other tumor biological parameters. The number of paired samples available for the analysis of the apoptotic index was higher in the E4 than in the placebo group and we did indeed detect a significant difference in favor of E4, although this result was not paralleled by expression data of the apoptosis-related proteins Bax and Bcl2.

Possibly, the most intriguing finding of this study is the effect of E4 treatment on intratumoral ER α and ER β . It is well known that estrogens can influence the expression of their receptors thereby modulating

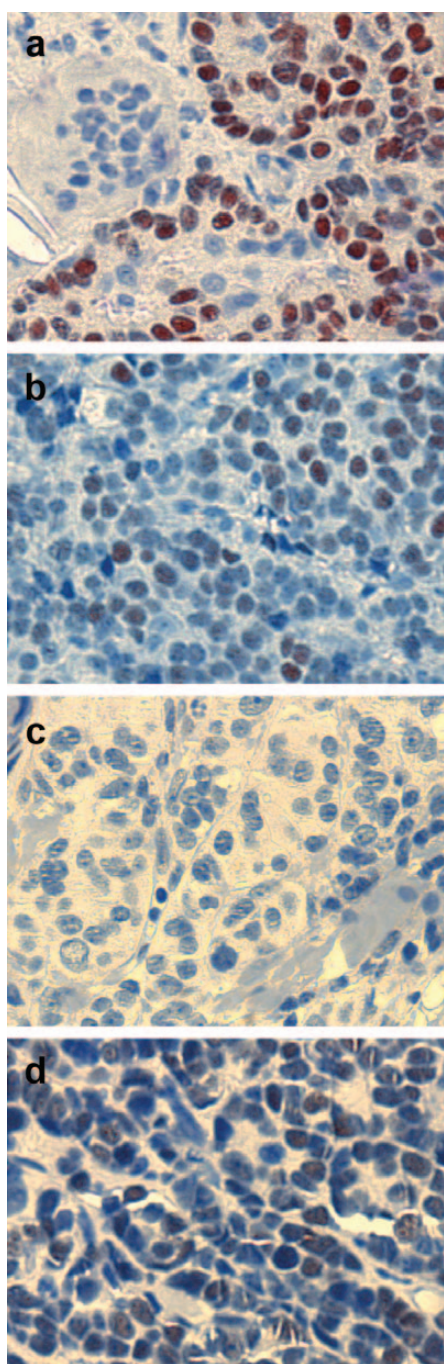


Fig. 4. Representative immunohistochemistry photomicrographs depicting ER α staining before (a) and after (b) 2 weeks of E4 treatment, and ER β staining before (c) and after (d) 2 weeks of E4 treatment.

their biological effects. Indeed, a 14 day treatment with E4 in this study significantly downregulated the ER α and showed a trend towards an upregulation of ER β . Several *in vitro* data support that ER β results in ER α antagonistic biological effects: when ER α and ER β are co-transfected into ER-negative cells, ER β inhibits ER α transcriptional activity and decreases the responsiveness of the cells to E2 (23,24). In HeLa cells transfected with ER α or ER β , ER β inhibits cyclin D1 gene transcription by E2, thereby providing further evidence for an antiproliferative effect of ER β (25). The effect of E4 on ER α and ER β may offer an explanation for the estrogen antagonistic effect of this fetal estrogen.

SHBG concentrations increased significantly during E4 treatment. Although similar effects can also be elicited by E2, the E2-induced

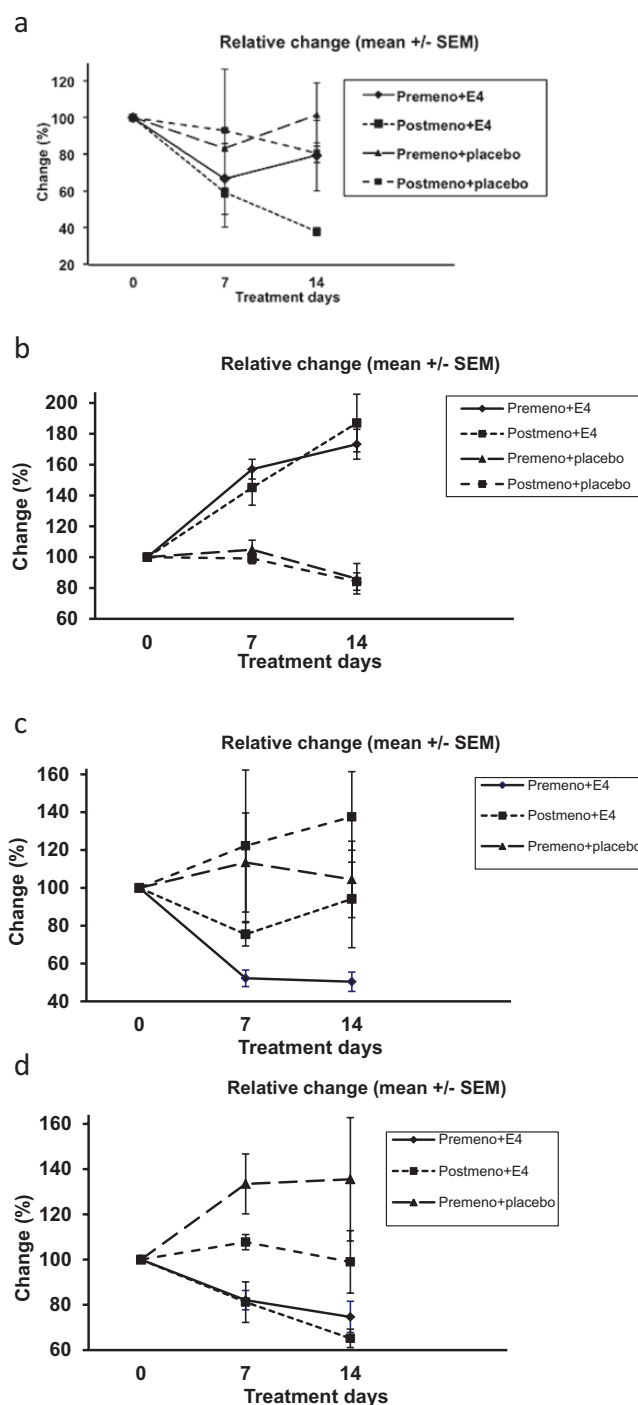


Fig. 5. Relative changes (%) in SHBG (a), FSH (b), bioavailable testosterone (c) and IGF-1 (d) serum concentrations after 14 days of treatment with E4 or placebo in pre- and post-menopausal BC patients.

upregulation of SHBG is part of a negative feedback loop. Because E4 does not bind to SHBG (4), the increasing SHBG levels reduce the bioavailability of other sex steroids such as free E2 and free testosterone, whereas E4 remains uncompromised in its bioactivity. This suggests a unique mechanism that selectively favors the bioactivity of a particular estrogen over others and which causes an overall low estrogenic environment.

IGF-1 is a highly mitogenic and antiapoptotic cytokine, which stimulates the growth of both normal and malignant breast epithelium (26). Because the IGF system represents a key growth regulatory pathway in BC, interfering with the IGF pathway might have a therapeutic potential

Table II. Mean serum concentrations (\pm SD) of endocrine parameters before (day 0) and during (day 7 and day 14) E4 treatment and 7 days after the last E4 dose (day 21) in pre- and post-menopausal women with early BC

Endocrine parameter		LH (U/l)	E2 (pg/ml)	Bioavailable E2 (pg/ml)	Testosterone (ng/ml)	Androstenedione (ng/ml)	Prolactin (ng/ml)	Progesterone (ng/ml)
Premenopausal + E4	Day 0	7.6 (3.2)	140.8 (115.1)	60.8 (35.7)	0.4 (0.2)	1.9 (0.6)	11.9 (5.2)	3.8 (4.6)
	Day 7	4.7 (2.0)	103.9 (55.2)	38.1 (20.1)	0.2 (0.1)	1.5 (0.5)	10.6 (3.0)	6.7 (5.6)
	Day 14	5.6 (3.0)	53.4 (55.3)	20.8 (20.7)	0.3 (0.1)	1.6 (0.5)	70.5 (95.9)	2.4 (2.9)
	Day 21	8.8 (3.0)	37.4 (21.7)	12.6 (10.1)	0.2 (0.1)	1.2 (0.5)	19.8 (26.3)	0.4 (0.1)
Postmenopausal + E4	Day 0	32.2 (7.9)	18.3 (3.9)	11.0 (1.2)	0.3 (0.2)	1.6 (0.4)	7.2 (2.8)	0.3 (0.1)
	Day 7	33.8 (9.7)	13.7 (2.9)	6.3 (1.8)	0.3 (0.2)	1.4 (0.4)	10.0 (2.8)	0.3 (0.1)
	Day 14	23.3 (14.5)	16.3 (3.3)	7.4 (2.6)	0.4 (0.3)	2.0 (1.3)	17.3 (10.7)	0.3 (0.1)
	Day 21	21.6 (6.4)	20.4 (11.7)	9.4 (6.3)	0.2 (0.1)	1.2 (0.4)	13.2 (5.8)	0.3 (0.2)
Premenopausal + placebo	Day 0	9.9 (3.9)	111.8 (113.9)	52.3 (56.6)	0.2 (0.1)	1.6 (0.9)	11.1 (1.5)	1.4 (1.4)
	Day 7	16.9 (30.2)	109.8 (33.2)	48.5 (16.3)	0.2 (0.1)	1.8 (0.6)	14.2 (6.0)	11.1 (7.2)
	Day 14	4.8 (1.1)	56.5 (21.0)	29.0 (14.3)	0.2 (0.1)	1.4 (0.4)	44.6 (67.7)	3.7 (6.1)
	Day 21	6.8 (3.3)	75.3 (47.2)	33.0 (20.9)	0.2 (0.1)	1.5 (0.4)	15.2 (8.9)	0.4 (0.2)
Postmenopausal + placebo	Day 0	25.5 (11.3)	16.5 (4.9)	7.5 (0.7)	0.3 (0.2)	1.6 (0.7)	7.1 (2.7)	0.3 (0.1)
	Day 7	26.6 (11.3)	13.0 (ND)	5.0 (ND)	0.3 (0.2)	1.2 (0.5)	5.6 (4.2)	0.3 (0.0)
	Day 14	24.6 (10.8)	14.0 (2.6)	7.0 (3.0)	0.3 (0.1)	1.2 (0.6)	18.8 (20.4)	0.3 (0.1)
	Day 21	26.8 (13.2)	15.5 (4.9)	6.5 (0.7)	0.2 (0.1)	1.2 (0.5)	11.8 (6.6)	0.2 (0.1)

in BC treatment (27). Our finding of a significant decrease in IGF-1 serum levels in response to E4 is surprising because estrogens are usually considered stimulators of the IGF system. This decline in systemic IGF-1 concentrations was not paralleled by a compensatory upregulation of intratumoral IGF-1R, which suggests a net reduction of IGF-mediated signal transduction.

This clinical data support the preclinical findings that E4 has antiestrogenic effects on BC cells, whereas earlier studies have shown that E4 has estrogenic effects on reproductive tissues and bone. Further clinical studies seem acceptable and are needed to confirm the safety and efficacy of E4 for the breast in hormone replacement therapy, including hormone replacement therapy in women who have or have had BC, especially in those BC patients treated with aromatase inhibitors and suffering from serious complaints due to estrogen deficiency.

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