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Different effects of tibolone and continuous combined estrogen plus progestogen hormone therapy on sex hormone binding globulin and free testosterone levels – an association with mammographic density

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Abstract

Objective To compare the effects of tibolone and continuous combined hormone therapy on circulating sex steroids and their binding proteins and their relationship to mammographic density.

Study design A prospective, double-blind placebo-controlled study. A total of 166 postmenopausal women were equally randomized to receive tibolone 2.5 mg, estradiol 2 mg/norethisterone acetate 1 mg (E2/NETA) or placebo. Serum analyses of sex steroids, insulin-like growth factor (IGF-I) and binding proteins and assessment of mammographic breast density were performed at baseline and after 6 months of treatment.

Results Estrogens were markedly increased and androgens decreased by E2/NETA. In contrast, tibolone had only a minor influence on circulating estrogens. Sex hormone binding globulin (SHBG) levels were reduced by 50%, while levels of androgens increased. Baseline values of estrone sulfate (E1S), around 1.0–1.1 nmol/l, were increased to 44.7 nmol/l by E2/NETA and to only 1.7 nmol/l by tibolone ($p < 0.001$). Mammographic breast density displayed a negative correlation with age and body mass index and a positive association with SHBG. After 6 months there was also a negative correlation with levels of free testosterone.

Conclusion We found that tibolone and E2/NETA caused distinct differences in estrogen/androgen status and blood levels of possible breast mitogens. The negative association between free testosterone and mammographic density could be a possible explanation for tibolone having less influence on the breast.

Keywords: *Hormone replacement therapy, tibolone, sex hormone binding globulin, free testosterone, mammographic breast density*

Introduction

After the early termination of the Women's Health Initiative (WHI) study, the long-term side-effects and risks of hormone replacement therapy (HRT) have been vividly discussed. HRT with combinations of estrogen and progestogen has been associated with an increased risk of breast cancer and deep venous thrombosis [1,2]. However, the interpretation of available data is complicated, since many different preparations, doses and regimens are used in clinical practice. HRT is not a uniform concept, and different compounds may well have different effects in this respect.

Tibolone has been suggested as an alternative to the common estrogen–progestogen therapy, and has been found to exert estrogenic, progestogenic but also androgenic effects in target tissues [3,4]. In previous studies tibolone was found to have less influence on the breast and a stronger influence on libido when compared to continuous combined estrogen–progestogen HRT [5,6]. Recently, tibolone was also been shown to have a different effect on the androgen status of postmenopausal women than continuous combined estradiol (E2)–norethisterone acetate [7].

Many of the effects of hormonal treatment reflect the change in levels of circulating steroid and peptide hormones and their binding proteins and as a consequence an altered response in various target tissues. For example, the increase in sex hormone binding globulin (SHBG) has been suggested to reflect the estrogenicity of the treatment at the hepatic level [8–10]. In young women using oral contraceptives, the relative increase in SHBG has even been suggested as a surrogate marker for the estrogen-dependent risk of thromboembolic disease [11].

Estrogen is a well-known mitogen in breast epithelium and several studies have shown breast cancer patients to have higher serum levels of estrogen than healthy women [12]. Cellular proliferation in the breast is stimulated by a series of paracrine and autocrine growth factors whose biosynthesis is enhanced by estrogens [13,14]. We recently reported a positive association between increased estrogen levels and breast cell proliferation during continuous combined HRT [15]. There are conflicting data on the association between androgen levels and breast cancer risk. Case–control studies have shown diverging results on testosterone levels in breast cancer patients compared to controls. In most prospective studies, however, no correlation between serum levels of testosterone, androstenedione, dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) and breast cancer risk has been found [16,17]. In cell cultures and animal experiments, androgens have been shown to excert antiproliferative effects [18,19].

Mammographic breast parenchymal density has been identified as an independent risk factor for breast cancer. The relative amounts of epithelium, stroma and fat in the breasts of individual women are clearly associated with age and body composition, but also with circulating hormones, growth factors and their binding proteins [20]. Recently we reported a prospective, randomized, double-blind placebo-controlled study where tibolone was found to have less influence on breast mammographic density than continuous combined HRT with estradiol and norethisterone acetate [5]. From this material, we here report the effects of treatment on circulating sex steroid and peptide hormones and their binding proteins, and their relationship to the mammographic density in individual women.

Materials and methods

Subjects

Apparently healthy postmenopausal women aged 50–70 years and without any previous history of breast disorder were recruited for the study. They were all postmenopausal for at least 12 months or had follicle stimulating hormone (FSH) levels of > 40 IU/l and estradiol levels of < 70 pmol/l. None of the women had taken any sex steroid hormones during the previous 3 months. Exclusion criteria were hypertension (systolic blood pressure > 170 mmHg or diastolic blood pressure > 105 mmHg), hyperlipidemia (total cholesterol > 8.0 mmol/l or triglycerides > 4.0 mmol/l) or type I or II diabetes mellitus.

A total of 166 women were randomized to receive either estradiol 2 mg /norethisterone acetate 1 mg (E2/NETA Kliogest®), tibolone 2.5 mg (Livial®) or placebo once daily for 6 months. The study medication was individually numbered for each subject.

Random assignment of subject code numbers over treatment groups was consecutively performed. Blinding was maintained until completion of the study.

Compliance was checked after 3 and 6 months from diaries where the women registered their daily medication intake. The women were not allowed to use any other sex steroids, warfarin, rifampicin, carbamazepine, griseofulvin, hydantoins, primodone or barbiturates during the study.

The study was approved by an independent Ethics Committee and all women gave their written informed consent prior to inclusion.

Analytical methods

Venous blood samples were drawn at baseline and after 6 months of treatment. Serum concentrations of testosterone and 17β-estradiol were determined by radioimmunoassay using commercial kits from Diagnostic Products Corporation, Los Angeles, CA, USA (Coat-a-Count®, testosterone) and from CIS Bio International, Gif-sur-Yvette, France (ESTR-US-CT®, estradiol).

SHBG, DHEAS and insulin-like growth factor I (IGF-I) were determined by chemiluminescence enzyme immunoassay using commercial kits obtained from Diagnostic Products Corporation (Immulite® SHBG and Immulite® DHEA-SO$_4$) and from Nichols Products Corporation, San Juan Capistrano, CA, USA (Advantage® IGF-I).

Insulin-like growth factor binding protein 3 (IGFBP-3) was analyzed by enzyme-linked immunosorbent assay using a commercial kit obtained from Diagnostics Systems Laboratories, Webster, TX, USA.

DHEA, 4-androstene-3,17-dione (A-4), unconjugated estrone (E1) and total estrone (tE1; $\geqslant 85\%$ estrone sulfate) were determined by radioimmunoassay after extraction with diethyl ether [21]. For tE1 estrone sulfate was hydrolysed by a *Helix pomatia* sulfataser plus glucuronidase preparation prior to extraction. Approximate values for estrone sulfate (E1S) were calculated by subtracting E1 from tE1 values. Values for tE1 above the highest point of the calibration curve, i.e., 50 nmol/l, were set at 50 nmol/l in the calculations.

Apparent concentrations of free testosterone were calculated from values for total testosterone, SHBG and a fixed albumin concentration of 40 g/l by successive approximation using a computer program based upon an equation system derived from the law of mass action [22].

The detection limits and within- and between-assay coefficients of variation were for testosterone: 0.1 nmol/l, 6% and 10%; E2: 5 pmol/l, 13% and 18%; E1: 30 pmol/l, 7% and 10%; tE1: 0.3 nmol/l, 7.0% and 8.9%; SHBG: 0.2 nmol/l, 6.5 % and 8.7%; A-4: 0.6 nmol/l, 6% and 10%; DHEA: 1.6 nmol /l, 5% and 7%; DHEAS: 0.002 μmol/l, 8.2% and 12%;

IGF-I: 6 µg/l, 5% and 7%; and for IGFBP-3: 0.04 µg/l, 9% and 10%, respectively.

Mammographic breast density

The effects of different treatments, i.e., E2/NETA, tibolone and placebo, on mammographic breast density have previously been reported [5]. In the present study we used a subset of data from this material of 154 women to assess the possible associations between density and circulating sex steroids, peptide hormones and their binding proteins. Mammograms were obtained at baseline and at 6 months and mammographic density was classified according to a percentage scale with five categories of the amount of dense breast parenchyma in relation to the whole breast volume. The five categories were 0–20%, 21–40%, 41–60%, 61–80% and 81–100% (Table I).

Statistical analysis

Differences between groups were tested by the Kruskal–Wallis test followed by post hoc analysis with the t test for unpaired observations or Mann–Whitney U test according to distribution. Changes from pre-treatment values were tested by the t test for paired observations or by the Wilcoxon signed rank test according to distribution. Correlations were assessed by Spearman's rank correlation and by multiple regressions. The significance level was set at $p < 0.05$.

Results

Of the 166 randomized women, 12 for various reasons, e.g., discontinuation of treatment and uncertain postmenopausal status, were not assessable. Thus, a total of 154 women, 48 on E2/NETA, 51 on tibolone and 55 on placebo treatment completed the study. There were no significant differences between the three groups with respect to age, body mass index (BMI), or years since menopause. Mean values were for E2/ NETA 57.1 years, 24.8 kg/m^2,

and 8.2 years; for tibolone 57.5, 24.5 and 7.9; and for placebo 57.2, 24.4 and 6.6, respectively. Serum hormone and protein levels did not differ at baseline.

The values at baseline and after 6 months of active treatment by E2/NETA and tibolone are shown in Table II. During placebo treatment there was no change from baseline values (data not shown). During treatment with E2/NETA there was a marked increase in circulating estrogens with a 10-fold rise in E2 ($p < 0.001$) and a near 20-fold increase in E1 ($p < 0.001$). Among women on tibolone no change in E2 and only a slight increase of E1 were recorded. In comparison, baseline levels of E1S around 1.0–1.1 nmol/l were increased to 44.7 nmol/l by E2/NETA and to only 1.7 nmol/l by tibolone ($p < 0.001$). While there was a significant increase in SHBG during E2/NETA treatment, levels of this binding protein were reduced by nearly 50% in women using tibolone. Levels of free testosterone were lowered in the E2/NETA group but significantly increased during tibolone treatment ($p < 0.05$). Adrenal androgens were virtually unchanged during treatment except for a slight decline in DHEAS during E2/NETA. After 6 months of treatment women on E2/NETA had lower values of IGF-I ($p < 0.05$) and IGFBP-3 ($p < 0.001$) than those on tibolone.

Mammographic status (Table I) at baseline displayed an inverse association with BMI (r_s _0.29; $p < 0.001$) and a positive association with SHBG (r_s 0.34; $p < 0.001$). After 6 months of treatment, these associations remained, and overall, there was also a negative correlation with free testosterone (r_s −0.27; $p < 0.001$). However, the negative association between density and free testosterone was only present in the tibolone group (r_s −0.28; $p < 0.05$) and the placebo group (r_s −0.35; $p < 0.01$). In contrast, the corresponding r_s value in the E2/NETA group was 0.04. Multiple regression after 6 months revealed SHBG as the most important factor ($p < 0.01$). Among women on active treatment ($n = 99$) after 6 months there was also a positive association between density and E1S (r_s 0.24; $p < 0.05$) and an inverse relationship with the E2/E1 ratio (r_s −0.29; $p < 0.01$). Also, in the placebo group there was a negative association between density and the E2/E1 ratio (r_s −0.46; $p < 0.01$).

Discussion

In clinical practice both E2/NETA and tibolone are frequently used to relieve vasomotor symptoms in postmenopausal women. In the present comparative study, we found distinct differences between these two regimens as regards their impact on blood levels of hormones, growth factors and binding proteins.

Treatment with E2/NETA resulted in increased estrogen and decreased androgen levels, in the latter case in total and free testosterone and DHEAS, which is in agreement with previous reports [23,24].

Table I. Data on mammographic status (number of women and percentage) according to percentage of dense breast parenchyma for postmenopausal women at baseline ($n = 154$) and after 6 months of treatment with either 17β-estradiol/norethisterone acetate (E2/NETA) ($n = 48$), tibolone ($n = 51$) or placebo ($n = 55$). Details on the effect of the different treatments were previously reported [5].

Percentage	Baseline	E2/NETA	Tibolone	Placebo
0–20	53 (34%)	7 (15%)	14 (28%)	18 (33%)
21–40	52 (34%)	10 (21%)	24 (47%)	14 (26%)
41–60	24 (16%)	20 (42%)	7 (14%)	11 (20%)
61–80	19 (12%)	9 (19%)	5 (10%)	8 (15%)
81–100	6 (4%)	2 (4%)	1 (2%)	4 (7%)

Table II. Serum levels of sex steroid and peptide hormones and their binding proteins in postmenopausal women before and after 6 months of treatment with either 17β-estradiol/norethisterone acetate (E2/NETA) ($n = 48$) or tibolone ($n = 51$).

	E2/NETA		TIBOLONE	
	Baseline	**6 months**	**Baseline**	**6 months**
E2 (pmol/l)				
mean ± SEM	24 ± 2	241 ± 17*** †††	26 ± 2	26 ± 4
median (range)	20 (6–87)	227 (54–546)	24 (8–57)	21 (5–157)
E1 (pmol/l)				
mean ± SEM	151 ± 8	2808 ± 194*** †††	179 ± 6	205 ± 11***
median (range)	139 (86–348)	3023 (635–5048)	181 (90–291)	206 (92–355)
E1S (nmol/l)				
mean ± SEM	1.0 ± 0.1	44.7 ± 1.2*** †††	1.1 ± 0.1	1.7 ± 0.2**
median (range)	0.9 (0.3–2.7)	46.8 (13.1–49.0)	0.9 (0.1–2.6)	1.2 (0.2–9.5)
E1/E1S ratio				
mean ± SEM	0.17 ± 0.01	0.06 ± 0.01*** †††	0.25 ± 0.04	0.20 ± 0.03*
SHBG (nmol/l)				
mean ± SEM	53 ± 2	64 ± 3** †††	50 ± 3	25 ± 3***
median (range)	55 (16–87)	62 (27–122)	47 (21–100)	22 (7–130)
T (nmol/l)				
mean ± SEM	0.8 ± 0.1	0.6 ± 0.1**	0.7 ± 0.1	0.6 ± 0.04**
median (range)	0.8 (0.2–3.0)	0.5 (0.1–1.6)	0.7 (0.1–2.1)	0.6 (0.1–1.2)
fT (pmol/l)				
mean ± SEM	15 ± 1	9 ± 1*** †††	14 ± 1	16 ± 1*
median (range)	14 (3–50)	8 (2–26)	13 (1–38)	16 (4–30)
A-4 (nmol/l)				
mean ± SEM	4.3 ± 0.3	4.1 ± 0.2	4.2 ± 0.2	4.4 ± 0.2
median (range)	4.0 (1.5–13.7)	4.3 (1.5–8.5)	4.0 (2.3–8.3)	4.5 (1.5–7.5)
DHEA (nmol/l)				
mean ± SEM	12.5 ± 1.2	11.3 ± 0.9	11.7 ± 1.0	13.4 ± 1.0
median (range)	10.0 (2.6–40)	10.0 (1.5–28.1)	10.6 (3.1–33.4)	12.2 (3.1–31.6)
DHEAS (μmol/l)				
mean ± SEM	2.5 ± 0.2	2.3 ± 0.2	2.6 ± 0.2	2.8 ± 0.2
median (range)	2.2 (0.5–8.1)	2.1 (0.5–6.7)	2.3 (0.5–7.6)	2.4 (0.5–8.3)
IGF-I (μg/l)				
mean ± SEM	117 ± 6	109 ± 5†	123 ± 6	127 ± 5
median (range)	104 (60–275)	107 (40–220)	120 (64–264)	126.5 (53–197)
IGFBP-3 (μg/l)				
mean ± SEM	3302 ± 125	3085 ± 100†††	3533 ± 138	3739 ± 153
median (range)	3141 (1818–5771)	2967 (1884–4892)	3500 (2084–6108)	3065 (2027–6837)

E2, 17β-estradiol; E1, estrone; E1S, estrone sulfate; SHBG, sex hormone binding globulin; T, testosterone; fT, free testosterone; A-4, 4-androstene-3,17-dione; DHEA, dehydroepiandrosterone; DHEAS, DHEA sulfate; IGF, insulin-like growth factor; IGFBP, IGF binding protein. Baseline vs. 6 months: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; E2/NETA vs. tibolone: †$p < 0.05$, †††$p < 0.001$).

These changes are characteristic for oral estrogen–progestogen HRT. It is well known that estrogens, when given orally, markedly influence liver metabolism and protein synthesis. After the administration of estrogen, dose-dependent changes are recorded in the circulating levels of a number of liver-derived proteins such as renin substrate, various coagulation factors and anti-thrombin [8,25]. There is also evidence that the type and dose of progestogen, when added to the estrogen, is important for liver metabolism and protein synthesis. Androgens and progestogens with androgenic properties are known to counteract some of the effects of estrogen on liver metabolism [26,27]. The dramatic decrease in free testosterone is mainly a result of the increased SHBG levels, and the slight decrease in DHEAS is likely to reflect the impact on the liver of oral estrogens, i.e., decreased circulating levels of its main binding protein albumin [28].

The picture following treatment with tibolone was quite different. There was only a minor influence on circulating estrogens, and SHBG levels were reduced by 50%. Androgens are known to suppress SHBG production at the hepatic level [9,10,26,27]. After oral intake, tibolone is rapidly converted into 3α- and 3β-hydroxy tibolone, both having estrogenic properties, and the Δ4 isomer, which is known to possess progestogenic as well as androgenic activity. In fact, the receptor affinity for this isomer is about 40% of that of the potent androgen dihydrotestosterone [3,4]. The marked reduction in SHBG levels and as a consequence increased concentrations of free testosterone implies an enhanced circulating androgenic activity. This may be important as regards some clinical effects of tibolone.

There are a number of observations to suggest that androgens may counteract the proliferative effects of

estrogen and progesterone in the mammary gland. Dimitrakakis and co-workers found treatment with flutamide (an androgen receptor antagonist) to markedly enhance breast epithelial proliferation in normally cycling rhesus monkeys [29]. Furthermore, in castrated animals, testosterone addition was found to inhibit breast proliferation as induced by estrogen and progestogen. Women with polycystic ovaries tend to have raised endogenous androgen levels and may also carry a lower breast cancer risk [30]. Androgen receptor dysfunction has been reported in some men with breast cancer [31]. Recently, a genetic linkage was suggested between androgen receptor dysfunction and BRCA-1 mutations [32]. Previously, in fertile women using oral contraceptives, we found an inverse relationship between circulating levels of free testosterone and breast tissue proliferation [33].

Recently we reported an increase in mammographic density in about 50% of women after treatment with E2/NETA, whereas only a few women (2–6%) had a similar response during treatment with tibolone [5]. Here, in the same material after 6 months of treatment there was an inverse relationship between mammographic density and free testosterone levels. There was also a positive association between density and SHBG levels, which was apparent at baseline as well as after treatment. Thus, one may suggest that the increased levels of free testosterone, together with the androgenic activity of the $\Delta 4$-isomer, may be one mechanism to explain why tibolone seems to have less influence on the breast than estrogen–progestogen HRT.

Decreased SHBG levels following treatment with tibolone have been reported previously and are believed to reflect an androgenic influence on the liver. However, it is not known whether this is due to the $\Delta 4$ isomer or to the hormonally less active tibolone molecule itself [4]. Thus, oral administration of epostane, a synthetic 17α-alkylated 5β-androstane derivative, which is devoid of any androgenic or anti-estrogenic potency, markedly alters serum levels of 'steroid-sensitive proteins' into an androgen-like pattern with low SHBG levels [34]. The capacity of a steroid to induce such changes may therefore be tied to its chemical structure rather than the intrinsic hormonal activity of the molecule.

Dören and co-workers previously found tibolone to increase DHEAS and suggested that this compound could modify adrenal androgen production [7]. Here, levels of the adrenal androgens A-4, DHEA and DHEAS were apparently unaffected after 6 months of treatment. While this could reflect the shorter duration of treatment, our data suggest that the principle androgenic functions of tibolone are exerted via altered liver metabolism.

It is widely accepted that estrogens are important for supporting the growth and evolution of breast cancer cells. Several authors have reported increased endogenous estrogen levels in postmenopausal wo-

men with breast cancer as compared to those without disease [12]. While there was a 10–30-fold increase in E2, E1 and E1S following treatment with E2/NETA, no change in E2 and only a slight increase in E1 and E1S were recorded among women on tibolone. There are data to indicate that tissue concentrations of E2 and E1S are significantly higher in malignant tumors than in normal breast tissue. Tibolone has previously been shown to inhibit the reduction of E1 into E2 by 17β-hydroxysteroid dehydrogenase as well as the hydrolysis of E1S into E1 by aryl sulfatase [35]. This effect was also evident in the present study, as indicated by reduced E2/E1 and E1/E1S ratios.

There is increasing evidence that the growth hormone–IGF-I axis is involved in carcinogenesis of the breast [13,14]. In fact, both SHBG levels and body composition, e.g., body fat distribution, may also be influenced by this regulatory system. Overall, both at baseline and after 6 months, we found lean postmenopausal women to have a higher breast density. Women with a high density also had higher SHBG levels. This finding may support epidemiological data suggesting that the increased risk of breast cancer during HRT is primarily related to women with a low or normal BMI [36].

Epidemiological studies have found increased levels of IGF-I and low levels of its major binding protein IGFBP-3 to reflect an increased breast cancer risk in premenopausal women [14]. IGF-I has been shown to stimulate breast cell proliferation, whereas IGFBP-3 has an inhibitory effect and also promotes apoptosis [13]. We found no significant changes during treatment with either E2/NETA or tibolone, but after 6 months IGFBP-3 was about 20% lower in women receiving E2/NETA as compared to those on tibolone.

In summary we found two alternative regimens for HRT in postmenopausal women to cause distinct differences with respect to estrogen–androgen status and blood levels of possible breast mitogens during treatment. In contrast to E2/NETA, tibolone displayed apparent androgenic properties. The negative association between levels of free testosterone and mammographic density could be one possible mechanism to explain why this substance may have less influence on the breast than conventional HRT.

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