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# Intratumoral levels of estrogens in breast cancer ☆

M.A. Blankenstein [a,*], J. van de Ven [a], I. Maitimu-Smeele [a], G.H. Donker [a], P. Chr de Jong [a], J. Daroszewski [b], J. Szymczak [b], A. Milewicz [b], J.H.H. Thijssen [a]

[a] *Departments of Endocrinology, University Medical Centre, Utrecht, The Netherlands*
[b] *Medical Academy, Wroclaw, Poland*

**Abstract**

Breast cancer tissue is an endocrine organ and particularly the estrogen biosynthetic properties of this tissue have been well studied. The concentration of estradiol in breast cancer tissue from postmenopausal patients is considerably higher than that in the circulation and appears to depend largely on local production. Androgenic precursor steroids are abundantly present, but estrogen storage pools like fatty acid derivatives appear to be less important than initially thought. New, potent and highly specific aromatase inhibitors effectively inhibit peripheral conversion of androgens to estrogens (Cancer Res. 53: 4563, 1993) as well as intratumour aromatase, median aromatase activity being 89% lower in the tissue from patients pretreated with aromatase inhibitor 7 days prior to surgery ($P < 0.001$). Also the intratissue concentrations of estrogens were decreased (64% and 80% reduction, respectively for estrone and estradiol; $P = 0.001$ and $< 0.05$; Cancer Res. 57: 2109, 1997). These results illustrate that intratissue estrogen biosynthesis is effectively inhibited by the new generation of aromatase inhibitors. The pathophysiological consequences of this finding are currently under study. © 1999 Elsevier Science Ltd. All rights reserved.

## 1. Introduction

There is no doubt that estrogens, and estradiol in particular, play a major role in the etiology of breast cancer. Estrogens may exert their facilitating action in the various steps leading to the development of clinically manifest tumours, i.e. from initiation to promotion and one of the major therapeutic approaches is aimed at interference with estrogenic action. This interference is exerted at two levels, i.e. by preventing the interaction of estrogens with the cells by blocking the estrogen receptor with anti-estrogens, or by interfering with the biosynthesis of estrogens through the administration of inhibitors of the enzymatic pathways leading to estradiol biosynthesis. As the action of aromatase is essential for the biosynthesis of estrogens, inhibition of this enzyme has been applied with relative success as a second line endocrine treatment. Although traditionally the administration of anti-estrogens has been the first line endocrine treatment, the use of this treatment modality in the adjuvant setting and even in prevention, has also advanced the use of aromatase inhibitors. Other developments have contributed to the increased popularity of aromatase inhibition and one of these is the development of aromatase inhibitors with considerably higher potency and specificity, combined with less side effects. Secondly, the observation that the breast is not a passive organ in which estrogenic effects are brought about by an external supply of estrogens, but is actively involved in estrogen biosynthesis may present new possibilities for therapeutic options. The purpose of this paper is to review our investigations on the accumulation of estrogens by cancerous and noncancerous breast tissues and possible means to interfere with this process. Attention will be focused on the levels of estrogens in various breast tissues, the activities of both aromatase and 17$\beta$-hydroxysteroid dehydrogenase (17-OHSD), the levels of estrogen precursors used as substrate by these enzymes, polar and apolar derivatives, and, finally, on

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* Corresponding author. Fax: +31-30-2505418.
*E-mail address:* m.a.blankenstein@lab.azu.nl (M.A. Blankenstein)

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the effects of aromatase inhibition on intratumoral aromatase activity and concomittant estrogen levels.

## 2. Materials and methods

To avoid unnecessary duplicature, materials and methods published elsewhere will only briefly be referred to when this is considered to be in the interest of the legibility of this paper. Methods not published in detail will, of course, be presented in full.

### *2.1. Patients and tissues*

The patients from which the tissues used to determine endogenous steroid levels and enzyme activities were obtained, as well as the methodology used for tissue processing have been described by Thijssen et al. [1]. The mastectomy specimens were separated into tumour, fatty tissue and normal glandular tissue by cold dissection by a trained pathologist. The various tissue fractions were minced at 0°C and stored in multiple aliquots at −80°C until further processing for assessment of steroid content or enzyme activity.

Patients participating in our study on the inhibition of peripheral aromatization were healthy postmenopausal volunteers, who ingested a single dose of the aromatase inhibitor Vorozole racemate and in whom the aromatisation of $^{14}$C-androstenedione was monitored [2]. Finally, the effect of a one week treatment period with Vorozole on intratissue estrogen levels and aromatase activity was evaluated in a group of 11 postmenopausal breast cancer patients scheduled for breast surgery [3].

### *2.2. Assessment of steroid concentrations in breast tissues*

The assessment of estrogen and androgen concentrations in breast tissue specimens has been described in detail [4,5].

Lipoidal estrogens in fatty tissues were assayed as follows. Five hundred milligrams of tissue were cooled in liquid nitrogen homogenized for 30–45 s with a Mikro-Dismembrator (B. Braun, Melsungen, Germany). The tissue was transferred to a vial containing 1.5 ml of 0.01 M phosphate buffer pH 7.5. After 15 min the mixture was centrifuged for 2 min at 1500 × g. The aqueous phase was removed from under the fatty layer by pasteur pipette and added to 20 ml ethanol/acetone 1:1 which contained 5000 dpm tritiated lipoidal steroid derivative to monitor for recovery. the lipoidal steroid derivatives were prepared by incubation of tritiated steroid with whole blood for 3 h at 37°C and were isolated by extraction with ethanol:acetone 1:1, delipidation with hexane: 70% methanol 1:1 and chromatography. Added to the fatty layer were 3 ml of ethanol:acetone. The contents of the tubes were vigorously mixed for 30 s on a vortex mixer immediately after the addition. The resulting fine emulsion was added to the aqueous phase with the tritiated steroid, the tube was rinsed with 2 ml of ethanol:acetone 1:1. The resulting mixture was centrifuged for 10 min at 2500 × g and 15°C after mixing from time to time for at least one hour. The resulting supernatant was decanted and evaporated to dryness under a stream of nitrogen at 37°C. The pellets were washed with a 2.5 ml of ethanol: acetone 1:1. The washings were added to the first supernatant. The residue was mixed with 70% methanol and stored overnight at −20°C to allow delipidation. Following centrifugation at 2200 × g for 10 min at 4°C the 70% methanol fraction was decanted and processed for conjugated and free steroids as described earlier [4,5]. The pellet was saponified for 2.5 h at 50°C in 2 ml of an 80:20 mixture of absolute ethanol and 40% KOH in distilled water. Vortex mixing was applied with intervals of 20 min. Following cooling to room temperature, 2 ml of distilled water and 4 ml of 8% sodium bicarbonate (w/v) were added and the ethanol was evaporated in 1 h at 37°C under a gentle stream of nitrogen. Liberated steroids were extracted from the remaining water phase twice with 6 ml of ethyl acetate, the combined extracts were evaporated to dryness under a nitrogen stream and the residue was reconstituted in 2 ml distilled water by gentle vortex mixing and allowing to equilibrate for 20 min after which the steroid were extracted with 2 × 5 ml of diethyl ether. The water phase was frozen in an ethanol-dry ice bath and the ether phase decanted. Following evaporation to dryness, the residue was dissolved in 1.0 ml of absolute ethanol. Recovery of trititiated lipoidal steroid was estimated and the remainder was used for chromatography and quantification. Recovery of lipoidal Pregnenolone, DHEA estrone and estradiol was 83 ± 3%.

### *2.3. Assessment of enzyme activities*

Throughout our studies, aromatase activity was estimated using the product identification assay following incubation of tissue homogenates with tritiated testosterone [1,3]. In vivo aromatase activity was measured by measuring the amount of $^{14}$C-estrone formed following the i.v. administration of $^{14}$C-Adione and $^{3}$H-Estrone, one third as priming dose, the remainder by infusion over a period of 2 h. Urine was collected for 4 days and estrone was purified and quantified. The percentage conversion was calculated as 100% × $^{3}$H:$^{14}$C administered/$^{3}$H:$^{14}$C in urinary $E_1$. The absolute conversion of androstenedione to oestrone was found

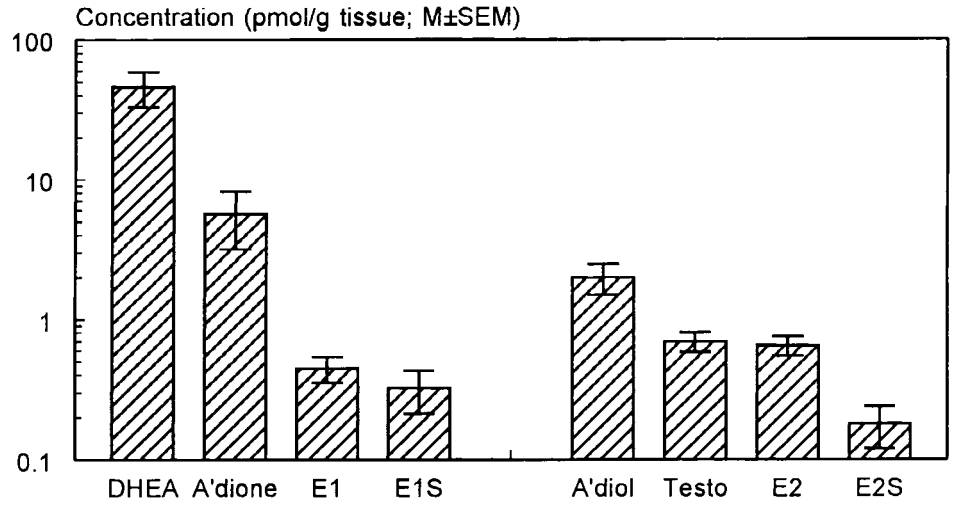


Fig. 1. Endogenous concentrations of androgens and estrogens in postmenopausal breast cancer tissue, indicating that breast cancer tissue has an adequate supply of estradiol precursors. Abbreviations: DHEA: dehydroepiandrosterone; A'dione: androstendione, A'diol: androstanediol, Testo: testosterone; E1: estrone; E2: estradiol; S: sulphate.

to be 2.19% [2], in excellent agreement with data published earlier by Reed et al. [6].

Activity of 17-OHSD was measured by determination of the conversion of radioactive estrone to estradiol, which was isolated and purified to constant specific activity prior to quantification.

## 3. Results

### 3.1. Steroids in tumour tissue

The concentration of estradiol in tumour tissue has been found to be independent of the plasma estradiol level [7–9]. This means that the plasma-to-tissue gradient for estradiol in breast tumour tissue is even higher for postmenopausal than for premenopausal women. This immediately raises the question as to the cause of estradiol accumulation in breast cancer tissue of postmenopausal women. One obvious mechanism would of course be through the action of the estrogen receptor (ER). Although in some studies, including our own, a statistically significant relationship between the concentrations of estradiol and ER can be shown, the observation that quantitatively significant estradiol levels are to be found also in ER-negative tumours [8] illustrates that other mechanisms must play a role. Of these, uptake from surrounding cells and tissue and in situ biosynthesis appear to be the most attractive. As the first possibility is extremely difficult if not impossible to approach experimentally, the possibility that the breast cancer cell provides in its need for estrogens by local synthesis has drawn considerable attention [10–12]. The observations that the breast has the capacity to express all the enzymes necessary for estrogen biosynthesis have lead to the recognition of the breast as an endocrine organ. Given the presence and activity of the estrogenic enzymes, the estrogen tissue level will be the result of biosynthesis and degradation. Apart from the enzyme activity, estrogen biosynthesis will also depend on the availability of substrate. Fig. 1 shows that the breast has no shortage of the precursor steroids androstanediol and testosterone, which directly lead to estradiol and DHEA and androstenedione which can contribute to estradiol biosynthesis via estrone.

### 3.2. Lipoidal estrogens

Based on a report by Larner et al. [13] fatty tissue, not only in the breast, but in other parts of the body as well, was considered a large reservoir of metaboli-

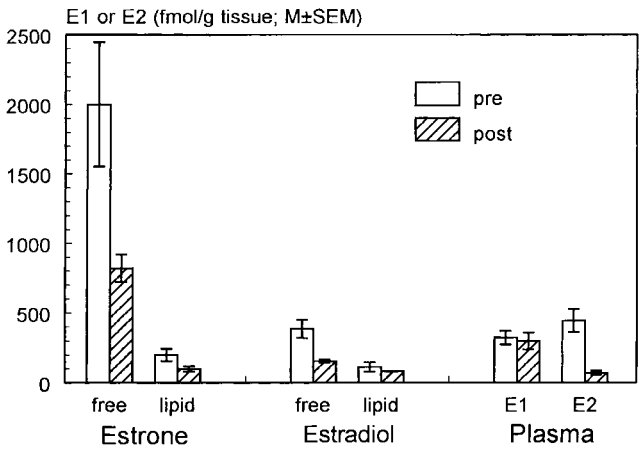


Fig. 2. Free and lipoidal estrogen concentrations in breast fatty tissues and plasma from pre- and postmenopausal women.

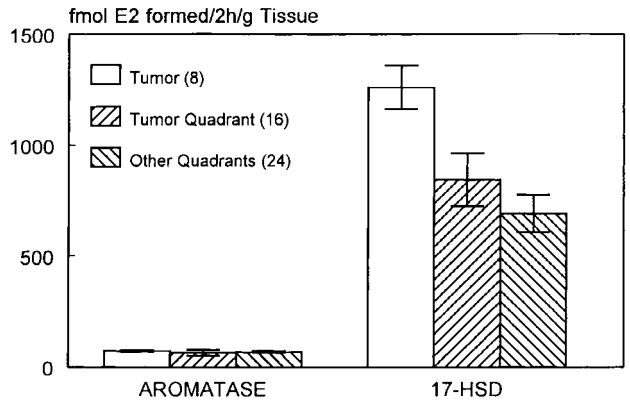


Fig. 3. Activities of aromatase and 17-hydroxysteroid dehydrogenase (17-HSD) in breast cancer tissue, in fatty tissue from the tumour quadrant, and in fatty tissue from tumor free quadrants of the mastectomy specimen.

cally active estrogen, i.e. estradiol-17-fatty acid esters. As adipose tissues from subcutaneous, internal and breast origin appear to be rather heterogenous [14] differences in steroid handling by the various fatty tissue might exist and we decided to quantitate the concentrations of lipoidal, free and sulphate-conjugated steroids in the various fatty tissues. The method used is described in detail in the Materials and methods section. Subcutaneous and omentum adipose tissue was obtained during abdominal surgery and breast adipose tissue during mastectomy for breast cancer. Concentrations of free and sulphate conjugated steroids in these tissue have been reported [15]. The concentrations of free and lipoidal estrogens in breast fatty tissue are shown in Fig. 2. There were no differences between the various fatty tissue investigated. It is clear from these results that the concentration of free steroids is considerably higher than that of the lipoidal derivatives. These results are in contrast to published results [13]. Our radioimmunoassay results have been confirmed by mass fragmentography, however, and therefore we conclude that the lipoidal estrogens are not the large estrogen reservoir they originally were thought to be and we submit that the compartment of lipid-conjugated steroid has no special role in steroid metabolism.

### 3.3. Steroidogenic enzymes

The activities of 17-hydroxysteroid dehydrogenase (17-OHSD) and aromatase are the activities leading directly to the formation of estradiol and hence these have been studied in great detail. For both enzymes, activity has been found to vary considerably between individual breast tumours. In general, 17-OHSD activity is much higher than aromatase activity (Fig. 3) and that is why in our opinion aromatase is the enzyme of choice for attempts to interfere with estrogen biosynthesis in the management of clinical breast cancer. Even if the activity of 17-OHSD could be inhibited by as much as 99%, there would still be enough substrate for the aromatase reaction.

### 3.4. Manipulation of enzyme activities

The observations that breast cancer is capable of maintaining its estradiol level even against the decrease in plasma estradiol associated with menopause and that aromatase is the rate limiting step in steroid biosynthesis justify attempts to decrease intratissue aromatase activity and explain the emphasis of the pharmaceutical industry on the synthesis of very active, highly specific aromatase inhibitors which cause considerably less side effects than the parent compound aminoglutethimide. Our experience in this field has been with Vorozole, both as a racemic mixture of two steroisomers, known as R76713, and as the pure active compound, the dextro-isomer Rivizor, R83842 [16]. A single oral dose of 1–5 mg, corresponding to 0.5–2.5 mg of the active compound was found to be very efficient in inhibiting peripheral conversion of androstenedione to estrone [2]. The degree of inhibition of peripheral aromatization obtained in this experiment ($93 \pm 2.5\%$ with a single dose of 1 mg Vorozole racemate) illustrates the potential of the new generation of aromatase inhibitors. It does not, however shed light into what happens in the tumour itself and therefore another experiment was conducted. In this experiment, patients with breast cancer scheduled for operation agreed to take 2.5 mg of Vorozole during 7 days prior to mastectomy. In the tissue removed, the activity of aromatase and the estrone and estradiol concentrations were determined and compared to those in tissues from women who did not take the drug. We found all three parameters to be significantly inhibited. Median tissue aromatase was 89% lower in women taking the aromatase inhibitor, whereas median estrone and estradiol were 64% and 80% lower, respectively [3]. This was the first report showing a concomittant decrease in intratissue aromatase activity and estrogen concentrations in breast cancer tissue following treatment with a nonsteroidal aromatase inhibitor. These results are in excellent agreement with those of Reed et al. showing [6] a significant reduction in normal and cancerous breast tissue estrone 36 h following a second of two intramuscular injections of 500 mg of 4-hydroxyandrostenedione, given 12 days apart. It should be borne in mind that both studies referred to have studied the effects of short term treatment. They provide 'proof of principle' and should be followed by long term clinical studies.

## 4. Conclusion

Based on our own results and data in the literature, it is safe to conclude that treatment of breast cancer patients with the newer aromatase inhibitors is a very effective way of depriving the tumour from its estrogens. No data on the effect of the first generation aromatase inhibitor aminoglutethimide on intratissue oestrogen concentrations are available and clinical studies will have to show whether the newer drugs are more effective in terms of response and survival. It has been reported however [10], that under treatment with aminoglutethimide breast cancer aromatase activity increases. Based on cell culture experiments with a human chorionic carcinoma cell line (JEG-3) it has been pointed out recently that one of the reasons why treatment with aminoglutethimide results in an increased aromatase activity in the tumour tissue [10] might be stabilization of the aromatase protein [17]. In the same cell line and human liver cells (HepG2), other aromatase inhibitors were also found to increase aromatase protein levels [18]. It remains to be established whether this phenomenon also applies to clinical breast cancer and, if so, whether it interferes with clinical efficacy of the treatment. Research on the regulation of intratissue estrogen levels will, therefore, continue and we hope to contribute significantly to developments in this area.

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