Pergamon

0960-0760(94)00124-3

J. Steroid Biochem. Molec. Biol. Vol. 51, No. 3/4, pp. 175–181, 1994

0960-0760/94 $7.00 + 0.00

Steroid Gradients Across the Cancerous Breast: An Index of Altered Steroid Metabolism in Breast Cancer?

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The concentrations of 17β-estradiol, estrone, testosterone (T), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate, androstenedione (A), cortisol and prolactin (PRL) were determined in the peripheral venous blood and in the lateral thoracic vein of 14 premenopausal and 34 postmenopausal women who underwent surgery for a breast carcinoma. The difference between the two blood samples, defined as concentration gradient across the cancerous breast, was calculated for all hormones. A significant peripheral–local concentration gradient was found for DHEA and A both in pre- and postmenopausal patients, whereas for T it was observed only in postmenopausal subjects. Furthermore, DHEA and A gradients were correlated to the presence of estrogen receptors as determined by a radioligand binding assay. An inverse relationship between DHEA gradient and the expression of estrogen receptors was observed in premenopausal women, whereas in postmenopausal patients an opposite, although not significant, trend was found. These results suggest that in the cancerous breast: (1) DHEA, A and T (the latter only in postmenopause) could be taken up from plasma, and thus there could be a storage of these steroids inside the breast tissue and/or perhaps some alterations in their local metabolism; (2) androgens could play a different role in breast carcinogenesis in relation to the estrogen circulating levels and to the expression of estrogen receptors.

J. Steroid Biochem. Molec. Biol., Vol. 51, No. 3/4, pp. 175–181, 1994

INTRODUCTION

Two decades ago, the demonstration of a subnormal urinary excretion of androgen metabolites in women who later developed breast cancer [1] first led to the hypothesis of a direct involvement of androgens in the pathogenesis of breast malignancies. Several authors speculated that androgens could exert a cancer-promoting action in the mammary tissue, and the following pathogenetic hypotheses were formulated:

(i) androgens could be a substrate for local conversion to estrogens, in turn co-responsible for the development of cancer [2]. Several experimental data were shown to support this view. First, the aromatase system was found to be active both in human mammary tumors [3, 4] and in the healthy tissue surrounding the breast cancer [5]. Moreover, aromatase activity was higher in quadrants harbouring breast cancer than in adipose tissue from unaffected quadrants [6]. Second, the conversion of androstenedione (A) and dehydroepiandrosterone (DHEA) to estrone (E1) and 17β-estradiol (E2) by human mammary carcinoma cells was observed *in vitro* [4, 7]. Finally, some steroids, among which was exogenous testosterone (T), were found to stimulate aromatase in different animal tissues [8].

(ii) Androgens could affect the local estrogen concentration by acting on the enzyme 17β-hydroxysteroid dehydrogenase (E2-DH), which catalyses the interconversion of E1 and E2. E2-DH was found to be active in ZR-75-1 human mammary cancer cells [9] and the inhibition of mammary E2-DH by DHEA, DHEA-sulphate (DHEA-S) and their metabolite androstenediol (Adiol) was demonstrated both *in vivo* [10] and in MCF-7 cell lines [11]. A reduced catabolism of E2 or an increased conversion of E1 to E2 could account for the high E2 concentration observed inside the mammary tumors [12].

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Received 18 Mar. 1994; accepted 5 July 1994.

(iii) Androgens could increase the mammary E2 concentration acting through the inhibition of E2-sulphotransferase activity. In fact, both DHEA and Adiol were reported to inhibit E2-sulphotransferase in breast tissue [13].

(iv) Androgens could act directly on the mammary tissue as co-factors in carcinogenesis. In fact, androgen specific binding sites were detected in the mammary gland [14], and Adiol was reported to bind both to them and to estrogen receptors, thus inducing estrogen-like biological responses [15] and modulating the interaction of E2 with the target cells [16, 17, 18].

Until now, the role of androgens in breast cancer was studied mainly by means of three experimental methods: (i) comparing their levels in the blood of women affected by the disease with those found in normal women, (ii) determining the androgen concentration in both cancerous and normal mammary tissues, or (iii) evaluating the activity of enzymes involved in androgen metabolism in cells derived from both normal and affected breasts. Unfortunately, all these approaches did not allow to fully clarify the androgen dynamics inside the breast. In fact, (a) steroid plasma levels may not reflect the tissue levels, (b) tissue androgen concentration is an unreliable marker of their transport into the breast and their local metabolism, and (c) enzymatic reactions can be deeply conditioned by the availability of substrates and co-factors, so that *in vitro* results may not reflect the *in vivo* situation.

In the present study, in order to evaluate the possibility of an active uptake by the mammary tissue and/or of a somehow altered steroid metabolism in the mammary gland affected by breast cancer, we determined the concentration of some steroids and prolactin in the peripheral venous blood and in the venous blood draining cancer-affected breasts.

MATERIALS AND METHODS

Patients

Forty-eight women affected by breast cancer were included in the study. Among them, 14 were premenopausal (age 35–49 years) and 34 postmenopausal for at least 18 months (age 53–79 years; mean ± SD time from menopause 11 ± 7.4 years).

Before surgery all patients underwent a thorough evaluation including mammary and axillary examination, breast *X*-ray, liver ultrasonography, total body scintigraphy and ultrasound-assisted fine needle aspiration followed by cytological examination.

None of the patients had a history of endocrine, metabolic or hepatic diseases, nor had they taken any hormonal treatment in the last 6 months.

All subjects underwent surgery in our Institution; a Pathey's mastectomy was performed in 12 cases, whereas in the other 36 the surgical intervention consisted of a tumorectomy with 2 cm-surrounding healthy margins associated with omolateral axillary limphoadenectomy. In all cases, an extemporaneous intraoperatory histological examination of the mammary node was performed in order to confirm the diagnosis of malignancy. Afterwards, a definitive complete histological examination was made with standard techniques, and its outcome always confirmed the intraoperatory exam. Breast cancer histotypes were as follows: ductal ($n = 39$), lobular ($n = 8$), anaplastic ($n = 1$). None of the breast cancers showed a multicentric origin.

Data regarding clinical characteristics of patients and breast tumors are reported in Table 1.

Blood samples collection and processing

All patients underwent a peripheral blood sampling some days before surgery in order to determine preoperatory hormone concentrations. In premenopausal women, this sampling was performed during the follicular phase of the menstrual cycle.

Intraoperatory blood samples were collected after the induction of general anaesthesia, at the time of axillary dissection. 10 ml of blood were taken simultaneously both from a peripheral brachial vein and from the lateral thoracic vein draining the affected breast, approx. 2 cm from its conjunction with the axillary vein. They were immediately carried to the laboratory and centrifuged (1500 *g*; 10 min.; room temperature); sera were stored at −24°C in 1 ml aliquots for 10–60 days until assayed.

Hormone assays

The following hormones were assayed in all blood samples: E2, E1, T, DHEA, DHEA-S, A, cortisol (F) and prolactin (PRL).

Table 1. Clinical characteristics of patients and breast cancers

	Premenopausal ($n = 14$)	Postmenopausal ($n = 34$)
Age (years)	35–49	53–79
Years from menopause (mean ± SD)	—	11 ± 7.4
Tumor histotype		
Ductal	13	26
Lobular	1	7
Anaplastic	0	1
Stage		
T1	4	15
T2	10	13
T3	0	5
T4	0	1
N+	7	13
N−	7	21
M+	—	—
M−	14	34
Estrogen receptors		
ER+	7	11
ER−	7	23

E2, E1, T and DHEA radioimmunoassays were performed using ^{3}H-tracers purchased from Amersham International (Amersham, UK). Highly specific antisera against E2-6-CMO-BSA, E1-3-HS-BSA, T-3-CMO-BSA, DHEA-17-CMO-BSA, 11α-hydroxy-A-11α-HS-BSA and DHEA-3-HS-BSA (for DHEA-S assay) were kindly supplied by Dr G. F. Bolelli (University of Bologna, Italy). Steroid standards for calibration curves were purchased from Sigma, (St Louis, MO, U.S.A.). E2, E1, T and DHEA were measured by RIA after solid-phase extraction with diethyl-ether on Extrelut 1 columns (Merck, Darmstadt, Germany), A after extraction with petroleum benzine, DHEA-S after sample dilution with Emagel (Behring, Marburg, Germany). The separation of free from antibody-bound steroid was performed after overnight incubation at 40°C using a dextran-coated charcoal suspension (10 min at 4°C). After centrifugation at 300 *g* for 10 min the supernatant ("bound" fraction) was assayed for radioactivity in a liquid scintillation counter (Wallac 1410, Turku, Finland) after addition of a liquid scintillation cocktail (Pico-Fluor 15, Packard Instruments Co. Meriden, CT, U.S.A.). For the mathematical processing of RIA data (dose–response curve fitting with a logistic model, calculation of results and evaluation of quality control parameters) the RIA-CALC software was used (Version 1.51, Wallac, Turku, Finland). The intra- and interassay coefficients of variation for all these assays ranged between 4.1–6.3 and 6.3–9.5%, respectively. F was measured by fluorescence polarization immunoassay on TDx (Abbott, Irvine, TX, U.S.A.; coefficients of variation: intraassay 2.3–3.7, interassay 3.1–5.5), PRL by time-resolved fluoroimmunoassay with a Delfia kit (Wallac; coefficients of variation: intraassay 4.1–5.8, interassay 5.0–6.7).

Receptor analysis

A fragment of each breast cancer was immediately frozen in liquid nitrogen and later used to perform estrogen receptor (ER) assay by a receptor binding method employing dextran-coated charcoal to separate bound from free radioactivity. This frozen tissue was pulverized in a microdismembrator at −80°C and then suspended in 10 vol of a cold balanced solution (K_2HPO_4 10 mM, KH_2PO_2 10 mM, K_2EDTA 1.5 mM, dithiothreitol 5 mM, sodium molybdate 10 mM; pH 7.4). The homogenate was centrifugated at 100,000 *g* for 90 min. To obtain the cytosol fraction, [^{3}H]E2 (0.5 nM, Amersham, Sp. act. 88 Ci/mmol) was used to label the receptors by incubating the cytosol preparation (total volume of the incubate 300 mcl) at 4°C for 18 h. The binding detectable in the presence of 500 nM cold E2 was considered as non-specific binding. Following incubation, 200 mcl of cold dextran-coated charcoal suspension (50 mg of dextran T70 plus 800 mg of a norit-A-charcoal in 100 ml incubation buffer) were added for 10 min to remove by absorption the unbound steroid. The preparation was then centrifugated at 3000 *g* for 10 min to precipitate the dextran-coated charcoal. 200 μl in duplicate were then added to 5 ml Biofluor scintillation cocktail (New England Nuclear, Boston, MA, U.S.A.) and counted in a β-counter. The results were calculated according to the method of Scatchard [19], expressing the amount of receptor sites as fmol/mg cytosol protein. The protein content in the cytosol was evaluated by the method of Bradford [20] using the BIORAD protein assay kit (Biorad Lab., Richmond, CA, U.S.A.). The lowest receptor concentration indicating a tissue as ER-positive was 6 fmol/mg cytosol protein.

Statistical analysis

Data were processed by STATGRAPHICS software [21] run on a IBM-compatible PC. The baseline vs the intraoperatory peripheral levels, as well as the latter vs the local concentrations of each hormone were compared using the Wilcoxon-Rank test for paired data. Hormone gradients in ER-positive vs ER-negative patients were compared by the Mann-Whitney test. The correlation between steroid gradients and some clinical characteristics, such as age, body weight and size of the tumour was checked by the Spearman's correlation analysis. The same test was employed to correlate the A gradient with the DHEA gradient as well as the latter with the surgery-related DHEA variation.

RESULTS

The blood DHEA and A intraoperatory peripheral concentrations were found to be significantly increased with respect to the preoperatory levels both in pre- and postmenopausal women, whereas T increased significantly during surgery only in postmenopause (Table 2). On the other hand, DHEA-S levels significantly decreased during surgery both in pre- and postmenopausal subjects. As expected, the surgery-related stress caused a significant increase of PRL and F in all patients. No significant differences between peripheral preoperatory and intraoperatory levels were observed for E1 and E2 (Table 2).

A statistically significant difference between intraoperatory peripheral and local hormone levels was observed for DHEA and A (Table 2). DHEA levels in the lateral thoracic vein were lower than in the general circulation in 13/14 premenopausal patients and in 33/34 postmenopausal women, whereas A was found to be reduced in thoracic vein blood in 12/14 premenopausal patients and in 33/34 postmenopausal subjects.

T concentrations in the mammary venous blood were significantly lower than in peripheral blood only in postmenopausal women, but not in premenopause (Table 2).

Table 2. Hormone blood concentrations before surgery (preoperatory) and during surgery in the brachial vein (peripheral) and in the lateral thoracic vein (local)

	Premenopausal			Postmenopausal		
		Intraoperatory			Intraoperatory	
	Preoperatory	Peripheral	Local	Preoperatory	Peripheral	Local
T (ng/ml)	0.47 ± 0.16	0.53 ± 0.18	0.54 ± 0.22	0.38 ± 0.16^{g}	$0.45 \pm 0.18^{h,i}$	0.42 ± 0.16^{l}
A (ng/ml)	1.5 ± 0.66^{c}	$2.6 \pm 0.76^{d,e}$	2.2 ± 0.90^{f}	0.82 ± 0.51^{c}	$2.2 \pm 0.73^{d,e}$	1.6 ± 0.63^{f}
DHEA (ng/ml)	5.9 ± 2.8^{a}	$14 \pm 5.1^{b,e}$	11 ± 4.9^{f}	2.6 ± 1.4^{a}	$7.5 \pm 3.2^{b,e}$	5.9 ± 2.8^{f}
DHEA-S (mcg/ml)	1.60 ± 0.62^{a}	1.15 ± 0.36^{b}	1.13 ± 0.42	0.83 ± 0.45^{a}	0.70 ± 0.39^{b}	0.72 ± 0.42
E1 (pg/ml)	79.1 ± 29.6	63.1 ± 26.2	58.3 ± 22.1	35.4 ± 14.3	39.9 ± 16.9	39.3 ± 15.4
E2 (pg/ml)	99.5 ± 34.3	86.7 ± 49.7	81.4 ± 49.7	20.4 ± 7.50	19.9 ± 7.89	21.7 ± 8.71
PRL (ng/ml)	15.5 ± 10.7^{a}	129 ± 80.2^{b}	127 ± 77.6	12.5 ± 19.5^{a}	109 ± 46.4^{b}	109 ± 44.7
F (ng/ml)	143 ± 46^{a}	646 ± 152^{b}	683 ± 173	127 ± 70^{a}	806 ± 192^{b}	798 ± 205

Significance levels: [a] vs [b] $P < 0.001$; [c] vs [d] and [e] vs [f] $P < 0.001$; [g] vs [h] and [i] vs [l] $P < 0.05$.

In premenopause, the mean of DHEA peripheral-distrectual gradients (ΔDHEA) was found to be significantly higher in ER-negative than in ER-positive subjects ($P < 0.05$) (Fig. 1). The same trend, although not statistically significant, was observed for the mean of A gradients (ΔA). On the other hand, in postmenopausal women ΔDHEA and ΔA were higher, although not significantly, in ER-positive than in ER-negative patients.

Considering both groups of patients together, the Spearman's correlation test between ΔDHEA and ΔA was statistically significant (Fig. 2), whereas there was no significant correlation between ΔDHEA and the surgery-related DHEA variation (Fig. 3). Both ΔDHEA and ΔA showed no correlation with the following variables: age of the patient, body weight, and the size of breast cancer (not shown).

No significant differences were found between the peripheral and distrectual levels of DHEA-S, E2, E1, F and PRL (Table 2).

DISCUSSION

Since 1971, when Bulbrook *et al.* [1] reported a subnormal excretion of androgen metabolites in women who later developed breast cancer, several experimental data led to the hypothesis that androgens could be directly involved in the development of mammary neoplasias. Nevertheless, neither studies about the circulating and/or tissue concentrations of androgens in affected women [12, 22], nor data concerning the activity of mammary enzymes involved in androgen metabolism [3, 4] permitted to fully clarify the role of androgens in the pathogenesis of breast cancer.

In the present work, we studied indirectly androgen metabolism in breast malignancies comparing the concentrations of some steroids in peripheral venous blood with those measured in the venous blood directly coming from the affected breast. Although steroid plasma levels may not reflect the true hormone concentrations inside the mammary tissue and thus may have

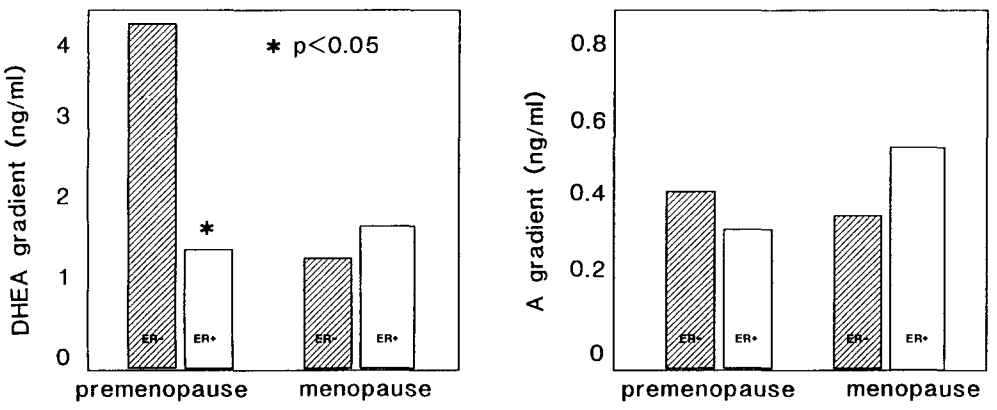

Fig. 1. Mean of DHEA and A peripheral–local gradients according to the expression of estrogen receptors (ER). In premenopausal women the mean of DHEA gradients was significantly higher in ER-negative ($n = 7$) than in ER-positive ($n = 7$) patients ($P < 0.05$). The mean of A gradients showed no significant difference in relation to ER expression. In postmenopausal subjects the opposite trend was observed both for DHEA and A (ER-negative: $n = 23$; ER-positive: $n = 11$), but it was not statistically significant.

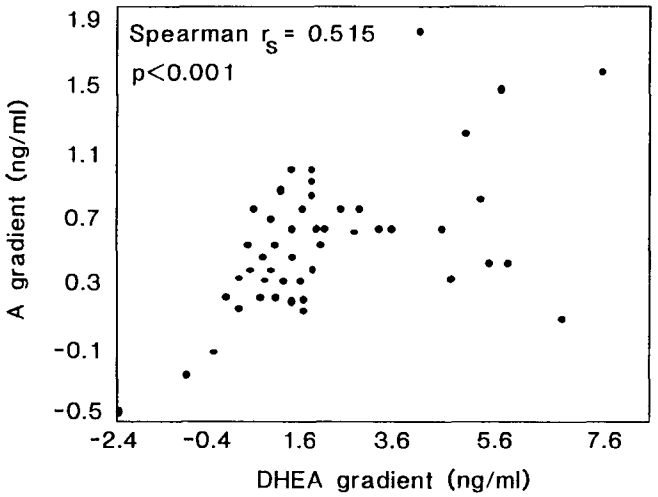

Fig. 2. Spearman's correlation between peripheral–local DHEA and A gradients. Considering all patients together, ΔDHEA and ΔA were significantly correlated ($P < 0.001$).

only an indirect biological significance, the measurement of the systemic–local concentration gradient is an original approach to look for the possible existence of a steroid uptake by the breast.

The relatively wide range of baseline hormone concentrations (expecially for androgens) that we observed can be explained by considering the marked variability in the age of subjects in both pre- and postmenopausal groups.

We noticed a significant increase of DHEA and A peripheral concentrations during surgery, in agreement with previous reports about the intraoperatory variation of circulating hormones [23]. However, this surgery-related increase should not have influenced the peripheral–local gradients that we measured for both DHEA and A; in fact, Spearman's analysis showed that DHEA gradient and the surgery-related DHEA variation were not correlated.

The significant decrease of DHEA that we observed in local compared to peripheral blood suggests the existence of an uptake of the steroid from plasma and its metabolism in the cancerous breast. This finding is in agreement both with the positive arterovenous gradient of DHEA shown by Deslypere [24] across the breast cancer tissue, and with the increased DHEA intratumoral levels previously reported by others [25, 26]. Moreover, the possibility of an uptake of some steroids, including DHEA, by breast cancer tissue was previously demonstrated [27, 28], and our group also reported a positive correlation between DHEA arterovenous gradient across the breast and its concentration inside the breast cancer [29].

The metabolic destiny of DHEA in the cancerous breast remains difficult to define. Estrogens did not increase in the thoracic vein blood, and this observation does not support the hypothesis of local aromatization of DHEA, even if it can not be excluded, since the estrogen tissue concentration in our patients is unknown. Also the conversion of DHEA to DHEA-S seems unlikely. In fact, some authors reported very low DHEA-S intratumoral concentrations and, to the contrary, showed the existence of an active conversion of DHEA-S to DHEA in the cancerous breast [25, 30]. Furthermore, in the present study we did not see an increase of DHEA-S in the blood draining affected breasts and, moreover, we previously failed to demonstrate in breast cancer a correlation between sulphatase activity and DHEA levels, as well as between DHEA and DHEA-S concentrations [29]. An alteration in the intracellular metabolism of DHEA and DHEA-S in breast cancer is also suggested by the finding that the tissutal concentrations of DHEA and its sulphate are correlated in normal breast, but not in the cancerous tissue [31]. According to the forementioned pathogenetic theories about the role of androgens in breast cancer, DHEA could also be converted to Adiol, and then interact with ER [15], or it could act directly inhibiting E2-DH [10] and/or sulphotransferase [13].

Of interest is that in premenopausal patients the amount of DHEA gradient was inversely related to the tumor ER content, as women with ER-negative cancers were shown to have significantly higher ΔDHEA when compared to subjects with ER-positive tumours. The inverse relationship between ER content and ΔDHEA, which was not observed in postmenopausal women, is probably related to the endocrine "milieu" and not to confounding variables, since ΔDHEA was not correlated either with age, or with body weight. The ER/ΔDHEA inverse relationship in women with normal estrogen circulating levels could suggest the existence of a sort of negative control exerted by estrogens on the mammary DHEA uptake/metabolism. In other words, in conditions of relevant local estrogen effect (ER-positive subjects) DHEA uptake would be less active. This control could even be part of a more

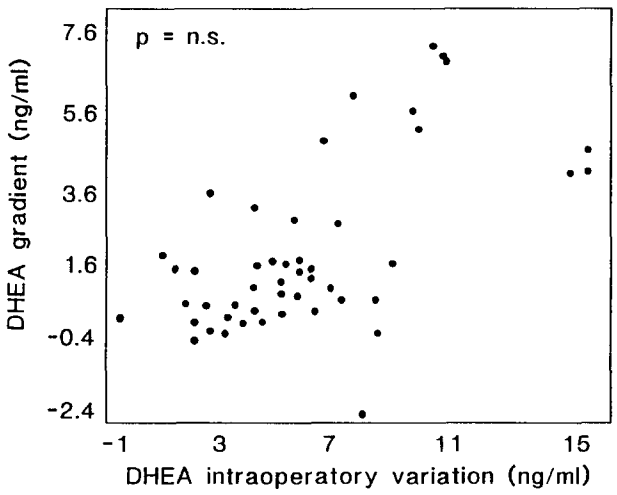

Fig. 3. Spearman's correlation between DHEA peripheral–local gradient and the surgery-related DHEA variation. Considering all patients together, ΔDHEA and the surgery-related DHEA variation were not significantly correlated.

complex paracrine interplay between adrenal androgens and estrogens inside the cancerous breast. It could be hypothesized that in premenopausal, ER-negative subjects androgens could have a direct role in carcinogenesis independently from their conversion to estrogens. The recent evidence that in the fatty tissue surrounding breast cancer there is not a tumour-directed gradient for androgens indicates that the aromatase activity, and consequently the maintenance of E2 concentrations inside the tumour, can be independent from the availability of androgen aromatase substrates [32]. On the other hand, in postmenopausal patients, when estrogen circulating levels are low, the existence of a higher ΔDHEA in ER-positive women could suggest a greater importance of androgen conversion to estrogens and thus a mainly indirect androgen effect on breast tissue.

Our observation of a significant peripheral–local venous gradient for A agrees with previous findings about the existence of a positive arterovenous gradient for this androgen across the cancerous breast [24, 33]. Since both the possibility of an uptake of A by breast cancer tissue and the existence of a significant correlation between plasma A and its concentration inside the cancerous breast were demonstrated [27], our finding supports the hypothesis that A is taken up from plasma by the mammary (cancer) tissue and then probably employed in several metabolic pathways: the metabolism of A could probably be related to that of DHEA, as suggested by the correlation between ΔDHEA and ΔA.

Although previous data about DHEA-S concentrations in women at high risk of breast cancer suggested the involvement of this steroid in the development of the disease, no differences in DHEA-S circulating levels between breast cancer cases and controls were reported [34]. In the present study, the absence of a significant DHEA-S gradient across the cancerous breast would suggest the lack of a DHEA-S uptake by the affected breast, according to the observations of Jones and James [26] and Vermeulen and Deslypere [27]. Anyway, this could be due to the high polarity of DHEA-S, which makes its entrance through the lipophilic cell membrane difficult. Moreover, the high DHEA-S circulating levels could mask small fluctuations of its concentration across the mammary tissue.

As far as the other steroids considered are concerned, we could only partially confirm the data of Deslypere [24] about the presence of an arterovenous gradient for T across the mammary cancer. In fact, we observed a significant gradient only in postmenopause. Moreover, we could not confirm the finding of Duvivier *et al.* [35], who signalled a positive gradient for E2 through the mammary gland. Anyway, in a previous study no significant correlation between T or estrogen plasma levels and their intratissutal mammary concentration was shown [27]. Furthermore, even if higher circulating levels of T were observed in women with breast cancer with respect to healthy subjects [36], no significant differences between T concentration in normal breast and tumour tissue could be demonstrated [37].

In conclusion, the study of the peripheral–local concentration gradients of some steroids, together with data coming from studies in which the intratissutal steroid levels and the activity of enzymes involved in their metabolism were considered, strongly support the hypothesis of a modified androgen transport and metabolism in the human breast affected by breast cancer. A better understanding of these phenomena could help to cast light on the hormone dependence of breast cancerous cells, the paracrine and autocrine mechanisms regulating their growth and the metabolic relationship between the tumour and the surrounding tissue.

Acknowledgements—This work was supported by the Grant No. 69/83, Ricerca Finalizzata Regione Piemonte. The authors wish to express their gratitude to Professor G. Giraudi, Dr B. Torchio and Dr D. Urbano for the precious technical assistance.

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