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Abnormal oxidative metabolism of estradiol in women with breast cancer

(endogenous estrogens/16α-hydroxylation/endocrine-related tumors)

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ABSTRACT **The three dominant oxidative biotransformations of estradiol were examined in 10 normal women and 33 females with breast cancer by using a recently devised radiometric method. Estradiol tracers, labeled with ^3H specifically in the 17α, C-2, or 16α position, were used to measure both the rate and extent of 17β-ol oxidation (the initial metabolic step) and the subsequent 2- and 16α-oxidative reactions. The mean ± SEM values for the extent of estradiol metabolism at these three specific sites were 76.9 ± 5.3%, 31.1 ± 4.0%, and 9.3 ± 0.8%, respectively, in normal subjects. Corresponding data in patients with breast cancer—i.e., 73.0 ± 4.2%, 32.7 ± 2.7%, and 14.9 ± 1.5%—revealed a significantly greater extent of 16α-hydroxylation in the latter population. Because the 16α-hydroxylated compounds (including estriol) are themselves potent estrogens, these changes may have important hyperestrogenic consequences that could have a bearing on the etiology of the disease.**

Endocrine factors have been implicated in the initiation or promotion, or both, of mammary tumorigenesis based on data collected from several sources. These include both experimental studies using animal models and epidemiological and clinical investigations in human subjects (1). Thus, certain features of the menstrual history and age at parity, which can lengthen the period of exposure to estrogens secreted by the ovaries, appear to be associated with an increased risk for the disease (2, 3). On the other hand, oophorectomy prior to age 35 can lessen the risk of breast cancer (3). Because an augmentation in estrogen secretion could be implicated in the etiology of the human disease, numerous studies have been conducted to detect such an increase in women with, or at high risk to develop, breast cancer (4). Although some investigators have reported significant differences in urinary or plasma estrogen levels, or both, between these subjects and normal individuals (5–7), these findings have not been consistent and have been challenged by others (8, 9). An alternative and possibly more viable hypothesis that an alteration in estrogen metabolism is associated with breast cancer (4, 10) has also been the subject of extensive investigations.

The metabolism of estradiol, which is primarily oxidative, consists of an initial oxidation of the 17β-hydroxy group to yield estrone. This steroid is subsequently metabolized mainly through either of two alternate hydroxylative pathways; namely, hydroxylation at the C-2 or the 16α position (11). These hydroxylations are of particular interest in that they constitute competing reactions whose products are themselves active compounds characterized by markedly different biological properties. The 16α-hydroxyestrogens—estriol and 16α-hydroxyestrone—demonstrate uterotropic activity comparable to that of the parent hormone, estradiol (12, 13). On the other hand, the principal 2-hydroxyestrogens—2-hydroxyestrone and 2-methoxyestrone—exhibit virtually no peripheral estrogenic effects but appear to play a regulatory role in neuroendocrine mediated events (14, 15).

Numerous studies have examined and compared the urinary estrogen metabolite patterns in women with breast cancer and normal subjects but these have yielded conflicting and inconclusive findings (4, 10). However, the results of these investigations were subject to inaccuracies inherent in the methods used, so that the issue of aberrant estrogen metabolism in breast cancer remained unresolved. The recent development of a novel radiometric procedure for assessing the oxidative metabolism of estradiol *in vivo* allowed us to reexamine the question by using a method not subject to some of the drawbacks associated with the prior techniques. In the current study, we have used this procedure to examine the three principal biotransformations of estradiol in peri- and postmenopausal breast cancer patients and in normal postmenopausal subjects. We report that breast cancer patients demonstrate a highly significant elevation in the reaction associated with the formation of the peripherally potent 16α-hydroxylated estrogens. A consequence of such an elevation would be a prolongation of the estrogenic effects of the endogenous hormone, estradiol, and can be viewed as being hyperestrogenic in nature.

MATERIALS AND METHODS

Subjects. Thirty-three patients with either primary or metastatic breast cancer (mean age, 58.5 yr; range, 43–74 yr) were studied. None were receiving hormonal treatment or chemotherapy at the time of the study. Two women had been treated by oophorectomy more than 2 yr prior to the studies, but none had undergone adrenalectomy or other ablative therapy. Estrogen receptor status was positive in tumor tissue obtained from 27 of these patients. Nine of the 33 patients were perimenopausal, having had their last menstrual period 6–12 mo previously. The remaining 24 patients were postmenopausal and had had no menses for at least 2 yr. The individuals studied had no history of liver, kidney, or endocrine dysfunction, and none had used oral contraceptives within the previous 2 yr. Fourteen of the subjects were receiving analgesics or mild antihypertensive medications, but these were discontinued several days prior to the estrogen metabolism studies. The results obtained in these individuals did not differ from those of the other patients. None of the subjects had taken drugs known to alter steroid metabolism within a 6-mo interval prior to the studies.

Ten normal women (mean age, 59.8 yr; range, 48–70 yr) were

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studied as controls. These subjects were postmenopausal by at least 2 yr, had previously had regular menses, were in good health with no history of liver, kidney, or endocrine dysfunction, and were taking no medications.

The racial and geographical backgrounds of the normal subjects and patients were similar, and all participants were within 90–135% of ideal body weight according to the Metropolitan Life Insurance Company tables.

Preparation of [2-^{3}H]Estradiol, [16α-^{3}H]Estradiol, and [17α-^{3}H]Estradiol Tracers. The [2-^{3}H]-, [16α-^{3}H]-, and [17α-^{3}H]estradiol tracers were prepared, and the specificity of the labels at the desired positions was confirmed as described (16). The substrate radiohomogeneity in the first two tracers exceeded 98%, and more than 95% of the ^{3}H was located at the designated position. For the [16α-^{3}H]estradiol tracer, substrate radiohomogeneity exceeded 96%, with 81% of the ^{3}H being situated in the 16α and 15% in the 16β orientation. Although oxidation of [17α-^{3}H]estradiol is accompanied by a substantial isotope effect, the rapid rate of the enzymatic reaction at this site minimizes its influence on the measurement of the extent of 17β-ol oxidation as determined by this radiometric method. Hydroxylation of estradiol at both the C-2 and 16α positions proceeds without an isotope effect (16), and a "National Institutes of Health shift" (17) does not occur in the case of the former. As recently shown (18), this provides for an essentially stoichiometric relationship between the amount of ^{3}H released and the extent of the oxidative reaction occurring at the C-2 position on the estradiol molecule. In the case of the [16α-^{3}H]estradiol tracer, a small contribution to the amount of ^3H_2O formed may be anticipated from the release of ^{3}H from the 16β position as a consequence of the formation of two minor estrogen metabolites—16β-hydroxyestrone and 16β,17β-triol. However, in view of the preeminence of the 16α-hydroxylated D-ring metabolites of estradiol in humans relative to the 16β metabolites, the extent of ^{3}H release from this tracer is likely to closely approximate the extent of 16α-hydroxylation *in vivo* (16).

Procedure. Labeled estradiol tracers were dissolved in sterile propylene glycol, and single dose aliquots were then stored in sealed ampoules. A measured weight of each tracer was administered as an intravenous bolus injection, and serial blood samples were drawn as described (16). For the [2-^{3}H]estradiol and [16α-^{3}H]estradiol studies, blood samples (10 ml for the former study and 15 ml for the latter) were obtained before and 1, 2, 4, 8, 12, 24, 36, and 48 hr after isotope administration. In this report the extent of 16α-^{3}H release in both populations was determined by using only those studies conducted for 48 hr after the administration of [16α-^{3}H]estradiol because it has now been established that this time interval is required to adequately measure the maximal extent of this reaction. In our initial studies in breast cancer patients, blood samples were obtained for only 24 hr, thus giving values which were submaximal. These early results were cited in connection with another investigation (19) and are not comparable to the present data. Blood (10 ml) was obtained before and 0.33, 0.67, 1, 2, 4, 8, 12, and 24 hr after administration of [17α-^{3}H]estradiol. When multiple tracer studies were performed, [16α-^{3}H]estradiol, [2-^{3}H]estradiol, and [17α-^{3}H]estradiol were administered sequentially over a 5-day interval. The three tracer studies were repeated in seven normal subjects to establish the reproducibility of the method. Analysis of the data by the paired Student *t* test revealed no significant differences between duplicate studies.

Data Analysis. Blood specimens were stored at −20°C for up to 96 hr, lyophilized, and the ^3H_2O thereby obtained was assayed in a Packard 2650 scintillation counter for a time sufficient for a counting accuracy of ±5%.

Total body water was measured in each individual by a ^2H_2O dilution procedure: 75 ml of 99.5% ^2H_2O (Merck) was administered orally to each subject, and a blood sample was obtained 3 hr later, at which time equilibration with total body water is achieved (20). The atom % excess of ^{2}H in the body water was determined by mass spectrometry, and these results were used to calculate total body water.

The percentage of oxidation at each time point after the administration of [2-^{3}H]-, [16α-^{3}H]-, or [17α-^{3}H]estradiol was then determined as the product of the plasma-water specific activity (dpm/liter) and total body water (liter) divided by the dose of ^{3}H-labeled steroid (dpm) administered, the fraction multiplied by 100. As in previous studies (16, 17), the time at which half of the maximal plasma-water specific activity was reached differed considerably for the 3 tracers (Fig. 1), with 17β-ol oxidation being the most rapid reaction. The turnover time of total body water is 9–13 days (20), which is relatively long when compared with the apparent rates of these oxidations *in vivo*. Because of this difference in rates, the maximal values for the percentage of oxidation, which occur within a 48-hr interval, correspond closely to the total extent of ^{3}H lost from each of the tracer steroids.

Statistical Analysis. The results are expressed as mean ± SEM values. Statistical analysis was performed by using Student's two-tailed *t* test; *P* values of <0.05 were considered to be significant.

RESULTS

The results of estradiol tracer studies using [2-^{3}H]estradiol, [16α-^{3}H]estradiol, and [17α-^{3}H]estradiol are illustrated in Table 1. The extent of oxidative metabolism at positions 17α, C-2, and 16α in the normal postmenopausal females were 76.9 ± 5.3%, 31.1 ± 4.0%, and 9.3± 0.8%, respectively. Corresponding values obtained for the patients with breast cancer were 73.0 ± 4.2%, 32.7 ± 2.7%, and 14.9 ± 1.5%, respectively. There was a statistically significant difference ($P < 0.01$) between these two populations for the biotransformation of estradiol at the C-16α position. Moreover, a plot of the data points for the maximal extent of 16α-hydroxylation in the control subjects and in the breast cancer patients demonstrates minimal overlap between the two groups with respect to this reaction (Fig. 1).

The time course for these three *in vivo* oxidative biotransformations were also plotted (Fig. 2). The percentage of 16α-hydroxylation (mean ± SEM) was significantly greater in breast cancer patients than in normal women when results were compared at 24, 36, or 48 hr after the tracer was given. The percentage of 17-oxidation, on the other hand, was significantly lower in the patient group at 1 and 2 hr after administration of this isotope, but the metabolic significance of this is unclear. The time at which the half-maximal percentage of oxidation was

Table 1. Maximal percentage oxidation of [2-^{3}H]estradiol, [16α-^{3}H]estradiol, and [17α-^{3}H]estradiol *in vivo**

Subjects	Estradiol metabolism,† % oxidation		
	2-Hydroxylation	16α-Hydroxylation	17-Oxidation
Breast cancer patients	32.7 ± 2.7 (24)	14.9 ± 1.5 (15)	73.0 ± 4.2 (13)
Normal women	31.1 ± 4.0 (10)	9.3 ± 0.8 (10)	76.9 ± 5.3 (10)
P	NS	<0.01	NS

NS, not significant.
* In breast cancer patients and normal controls.
† Mean ± SEM; the number of subjects studied is shown in parentheses.

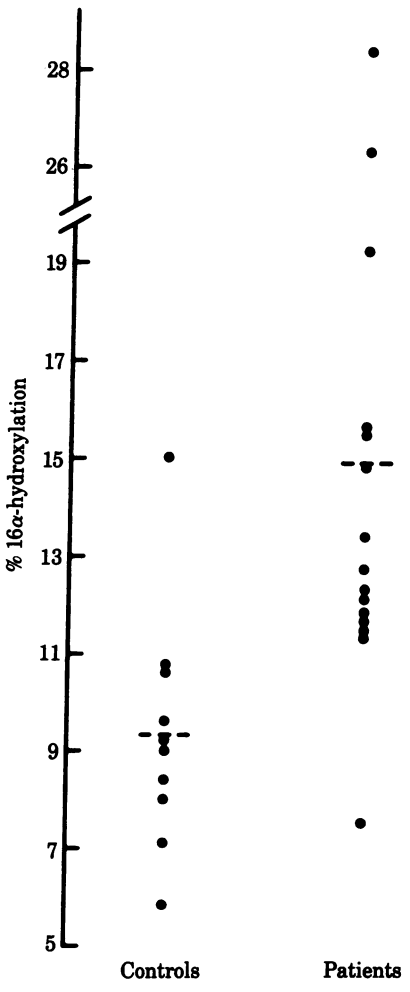

FIG. 1. Maximal [16α-^{3}H]estradiol oxidation in 10 control women and in 15 patients with breast cancer. Values expressed are ^3H_2O formed as a percentage of the administered dose. Means are represented by horizontal dashed lines.

reached after the administration of these tracer steroids was calculated for the two groups of subjects (Table 2) and did not differ significantly for the site-specific 17- and 2-oxidations, with the apparent slower oxidation at 16α in the breast cancer patients just escaping significance. The absence of any such trend in the other two reactions indicates that a decrease in metabolic clearance rates is not involved in the observed changes.

The estrogen metabolism results in the breast cancer patients were analyzed also on the basis of estrogen receptor data (i.e., positive vs. negative), both the duration and extent of disease [i.e., stages I and II vs. stages III and IV, according to the TNM classification (21)], and duration of response to treatment (i.e., <6 mo or >8 mo). No statistically significant differences were apparent when these variables were examined (data not shown).

We performed at least two radiometric studies in 19 breast cancer subjects and all three studies in each of the normal subjects. In patients available for two studies, we gave priority to the [2-^{3}H]- and [16α-^{3}H]estradiol tracers because of the distinctive biological properties of the products of these competing pathways. Moreover, the ratio of 2- vs. 16α-hydroxylation in a given individual would not be affected by the extent of 17β-ol oxidation because the latter is the initial step in the oxidative biotransformation of estradiol, preceding both 2- and 16α-hydroxylations, and, therefore, would affect both to a similar degree. The mean ± SEM values for the ratio of 2- vs. 16α-hydroxylation given in Table 3 for subjects undergoing both studies indicate that this ratio is significantly diminished in the breast cancer patients (2.2 ± 0.4 in the cancer patients and 3.5 ± 0.5 in normal subjects, $P < 0.05$). This finding is consistent with an absolute increase in 16α-hydroxylation in these patients.

DISCUSSION

There is evidence from several sources to suggest that estrogens have a positive etiologic or permissive role in breast cancer. The breast is normally an estrogen-responsive tissue, and breast tumors are often treated effectively with hormonal ablative therapy (22, 23). Certain factors consonant with either prolonged or excessive estrogen exposure appear to increase the risk for the development of the disease (1–3, 24). In addition, mammary tumors can be produced in rodents by the administration of exogenous estrogens (25, 26).

An argument can be put forth that the relative amounts of specific estrogen metabolites rather than the quantity of the secreted parent substrate increase the risk for the disease, either by prolonging estrogenic activity or by virtue of the unique biological properties of a particular metabolite. Previous studies, which examined estrogen metabolism in normal subjects and breast cancer patients by analysis of urinary metabolites, failed to establish consistent and clear-cut differences between these populations (4). Two methodological problems that might account for some of the discrepancies in the data include the differences in the analytical procedures used and the variability in the fraction of urinary estrogen metabolites accessible for measurement (4, 27). Estrogens are particularly subject to changes in excretory routes and conjugative pathways because of the fact that they undergo extensive enterohepatic circulation. The radiometric technique employed in the current study provides a number of advantages, as noted (16). A particularly attractive feature of this method is that it measures the total extent of *in vivo* metabolism of estradiol at specific ^{3}H-labeled sites on the steroid molecule and is independent of both further biotransformations or excretory routes (16).

We have examined the three major oxidative biotransformations of estradiol in 33 women with breast cancer and 10 normal subjects by using specifically radiolabeled substrates. The results of this study demonstrate that individuals with breast cancer have a significant 50% increase in metabolism at the 16α position compared to matched controls. Since the 16α-hydroxylated metabolites derived from this biotransformation show fully potent estrogenic effects (12, 13, 28), our findings suggest that this alteration in estrogen metabolism manifested in breast cancer acts to extend the *in vivo* estrogenic activity of the endogenous hormone in these individuals. Alternatively, the 16-oxygenated metabolite(s) could possess unique biological properties that translate into a greater risk for the disease.

Examining estrogen metabolism in a number of other diseases by the radiometric method, we have identified only systemic lupus erythematosus (SLE) as being associated with increased oxidation at the 16α position (19). Comparison with the present study is inappropriate because all of the women subjects and controls in the systemic lupus erythematosus study were premenopausal. The increased oxidation at the 16α position in the women with systemic lupus erythematosus was accompanied by a corresponding decrease in oxidation at C-2, a feature absent in the breast cancer patients, suggesting that both the causes and consequences of the metabolic alterations in the two diseases are different.

The estriol hypothesis has been a dominant theme in the search for a link between estrogen metabolism and breast can-

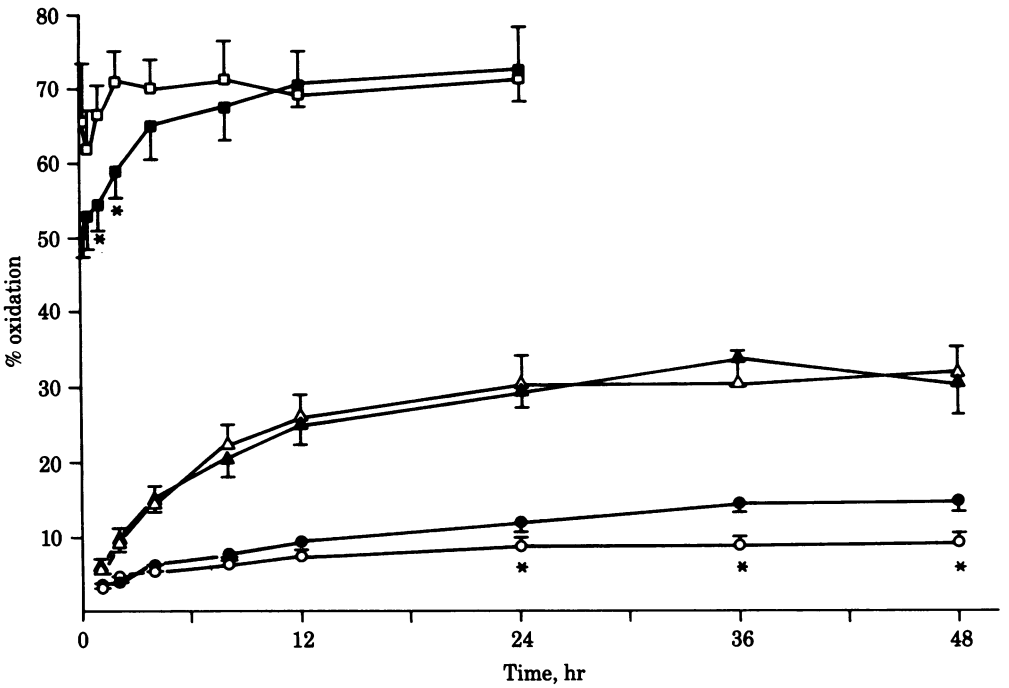

FIG. 2. Comparison of the percentage of oxidation of [2-^{3}H]-, [16α-^{3}H]-, and [17α-^{3}H]estradiol *in vivo* in normal controls (open symbols) and breast cancer patients (solid symbols) at timed sample-collection points. ○ and ●, 16α-hydroxylation; △ and ▲, 2-hydroxylation; □ and ■, 17-oxidation. Values are shown as mean ± SEM. *, Significant difference between the two groups.

cer. This hypothesis, which states that an increase in estriol formation relative to estrone and estradiol is associated with a decreased risk for the disease, was formulated both on the basis of the established protective effect of early pregnancy, a condition characterized by elevated estriol levels, and the fact that estriol was regarded as an impeded estrogen (7, 29). Although some investigators provided epidemiological evidence to support this theory (30–33), others reported contradictory results (10, 34–36). Moreover, a recent evaluation of estriol pharmacology has established that this steroid behaves as a potent estrogen agonist under physiological conditions (12, 13, 25).

The present work in which we demonstrate an elevation in 16α-hydroxylation in breast cancer fails to support the estriol hypothesis whereby a diminution in this reaction would be required in the affected individuals. It could be argued that the increase in 16α-hydroxylation observed by us could represent only enhanced formation of 16α-hydroxyestrone and, hence, might still be compatible with a decrease in the urinary excretion of estriol. However, prior work from this laboratory conducted on men and women with breast cancer demonstrated an elevation in the urinary excretion of radiolabeled estriol after the administration of a tracer dose of estradiol (10, 27). This would suggest that estriol formation represents at least a fraction of the increase in 16α-hydroxylation measured by the radiometric procedure; therefore, the present results are not in accord with the estriol hypothesis. However, the present work does support the concept that an alteration in the metabolism of endogenous estrogens may play a role in breast cancer because the 16α-hydroxylated compounds (i.e., 16α-hydroxyestrone and estriol) are potent estrogen agonists with minimal affinity for the plasma carrier protein, the sex hormone-binding globulin (SHBG) (28). The latter feature ensures that their biological activity, as compared to that of the other estrogens, would be greatly disproportionate to their circulatory levels because of the absence of any modulation by the sequestering carrier protein. Recently, evidence has been obtained that 16α-hydroxyestrone, one of the two 16α-hydroxylation products, forms covalent adducts with primary amino groups by a nonenzymatic process (unpublished data). Such interactions with macromolecules provides an intriguing mechanism for the possible participation of this metabolite in the oncogenic process.

A serious difficulty with any attempt to relate the endocrine profile of postmenopausal women with breast cancer to the onset of the disease is the probability that the initiation of the can-

Table 2. Time intervals required to attain half-maximal percentage oxidation of [2-^{3}H]estradiol, [16α-^{3}H]-estradiol, and [17α-^{3}H]estradiol *in vivo**

Subjects	Time for half-maximal oxidation, hr		
	2-Hydroxylation	16α-Hydroxylation	17-Oxidation
Breast cancer patients	5.0 ± 0.4 (24)	7.1 ± 1.1 (15)	0.3 ± 0.01 (13)
Normal women	4.7 ± 0.6 (10)	4.3 ± 0.8 (10)	0.2 ± 0.02 (10)
P	NS	NS	NS

Values and abbreviations are as in Table 1.
* In breast cancer patients and normal controls.

Table 3. Comparison of the extent of 2- and 16α-hydroxylation of estradiol *in vivo**

Subjects	Estradiol metabolism, % oxidation		
	2-Hydroxylation	16α-Hydroxylation	Ratio 2/16
Breast cancer patients	32.3 ± 4.9 (11)	16.5 ± 1.8 (11)	2.2 ± 0.4 (11)
Normal women	31.1 ± 4.0 (10)	9.3 ± 0.8 (10)	3.5 ± 0.5 (10)
P	NS	<0.005	<0.05

Values expressed and abbreviation are as in Table 1.
* In those breast cancer patients and normal women undergoing both studies.

cer preceded its clinical manifestation by a lengthy time interval (24). For this reason measurements of urinary or plasma levels of the parent hormones, such as estradiol and estrone, in postmenopausal women with breast cancer may have little bearing on the disease onset as they would not reflect hormonal status during the premenopausal period when the initiation of the disease occurred. On the other hand, it might be expected that the enzymatic pattern of estrogen metabolism in women with breast cancer would not change as a function of age. Supporting evidence for this is provided by the similarity in the extent of 2- and 16α-hydroxylation of estradiol in normal, pre- and postmenopausal women as determined with the radiometric procedure (16). Therefore, such radiometric measurements might provide a more accurate assessment of the hormonal pattern present at the time of tumorigenesis, which could be connected with the etiology of the disease. At present, it remains to be determined whether the increase in 16α-biotransformation demonstrated in breast cancer patients is a preexisting condition or is secondary to the disease process. Studies are currently in progress to assess whether women who are at high risk for familial breast cancer, but who have not yet developed the disease, have a similar alteration in estrogen metabolism. Changes in the conjugative pattern of urinary estrogen metabolites have been detected in these individuals (5), and it is possible that these may be associated with an elevation in estradiol 16α-hydroxylase activity. If it can be shown that this metabolic distortion precedes the clinical manifestation of the disease, the increased biotransformation at the 16α position of estradiol could serve as a useful risk marker for breast cancer. In addition, the identification of specific disease-linked abnormalities in estrogen metabolism would provide a new framework for the analysis of the mechanisms of estrogen participation in breast tumor initiation and promotion.

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