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It has been suggested that women who metabolize a larger proportion of their natural estrogen via the 16α-hydroxy pathway may be at significantly elevated risk of breast cancer compared to women who metabolize proportionally more estrogen via the 2-hydroxy pathway. This study evaluates whether the ratios of 16α–OHE1 to 2-OHE1 are higher in urine of postmenopausal breast cancer cases than in controls; and whether the ratio is elevated in cases independent of total urinary estrone (E1), estradiol (E2) and estriol (E3). Early morning urine samples are collected from 100 breast cancer cases and 100 controls who are participating in an ongoing case-control study of breast cancer at our institution. Five estrogen metabolites in urine are determined: 16α–OHE1, 2-OHE1, E1, E2 and E3 conjugates. The data collection is in progress.

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(4) Table of Contents:

(5) Introduction:

There is overwhelming evidence for a role of ovarian hormones in the etiology of breast cancer (1). At menopause there is a sharp decline in the amount of circulating estrogen, and this decline is at least part of the explanation for the decreased risk associated with early menopause (2). In postmenopausal women, the major source of estrogen arises from the peripheral conversion of androstenedione in adipose tissue (3). This is the most probable explanation for the increased risk of breast cancer associated with obesity in postmenopausal women (4). Elevated serum estrogen levels have been associated with an increased risk of breast cancer in postmenopausal women (5-16). Increased urinary excretion rates of E1, E2 and estriol (E3) have also been found in breast cancer cases as compared with controls (17-24).

The extent to which E2 is metabolized via the 16α-hydroxylation pathway may also be associated with breast cancer risk (25-27). The two main pathways for metabolizing E2 are via 16α-hydroxylation and via 2-hydroxylation of E1. The 16α-metabolites are biologically active (28, 29); the 2-hydroxy-metabolites are not (30). Data demonstrating the difference in biologic activity between 16α-OHE1 and 2-OHE1 are shown in table 1 (29). Continuous administration of these metabolites to ovariectomized rats resulted in a large weight increase in rats receiving 16α-OHE1 or estradiol, but very little change in rats receiving 2-OHE1.

Table 1. Effect of continuous estrogen (1 ug/h) in ovariectomized rats on wet uterine weights as percent of control (mean % ± s.e.) (29).

	Time after implantation		
	24h	48h	72h
Estradiol	165% ± 15%	363% ± 20%	490% ± 22%
16α-OHE1	155% ± 7%	365% ± 16%	552% ± 42%
2-OHE1	98% ± 1%	124% ± 1%	130% ± 9%

Increased 16α-hydroxylation, but not 2-hydroxylation activity has been observed in mice strains with high spontaneous mammary tumor formation (25). The extent of biotransformation of ^{3}H-labeled E2 via the 16α-hydroxylation pathway was found to be 4.6-fold higher in terminal duct lobular units (TDLUs) in breast tissue from breast cancer cases than in breast tissue from reduction mammoplasty controls (31).

The epidemiologic data that address this hypothesis are sparse. Schneider, Bradlow, Fishman and their colleagues injected 33 peri- and postmenopausal breast cancer patients and 10 postmenopausal controls with ^{3}H-labeled estradiol. They found a 60% higher extent of 16α-hydroxylation among the cases; this difference was statistically significant. There was, however, no statistical difference in 2-hydroxylation between the two groups; 2-hydroxylation was only 5% higher among cases. The ratio of the average level of 16α-hydroxylation to the average level of 2-hydroxylation was 52% greater in the breast cancer cases than the controls (32). This is the only published study that has examined this aspect of estrogen metabolism and breast cancer risk among postmenopausal women. No data on total estrogen values, dietary or non-dietary risk factors were provided.

The only other study of 16α-/2-hydroxylation in breast cancer patients was performed by Adlercreutz (33); this study is difficult to interpret. They examined estrogen metabolites in young

premenopausal breast cancer cases (n=10) and controls. The controls were women on an "omnivorous normal Finnish diet" (n=12) or lactovegetarians (n=11). There was no statistical significant difference in total urinary estrogens, 16α-hydroxylation, 2-hydroxylation or 16α-/2-hydroxylation between breast cancer patients and omnivores or breast cancer patients and lactovegetarians. The omnivorous women had, however, a higher fat intake than breast cancer patients. Because fat intake has been associated with increased 16α-hydroxylation (38), this could explain why no differences were found between the omnivores and the cancer patients, but it does not explain the lack of difference between the cancer patients and the vegetarians. The cases and controls were not comparable on parity: 7 of the 10 breast cancer cases, but only 6 of the 23 controls had ever given birth. Whether parity influences the ratio of 16α-/2-hydroxylation is unknown.

Both of the above mentioned studies measured metabolites after breast cancer diagnosis. Thus there is the possibility that the results obtained in one or both of these studies may have been affected by the cancer or the cancer treatment. In an attempt to determine whether an elevated ratio of 16α- to 2-hydroxylation precedes diagnosis, Osborne, Bradlow and coworkers studied estrogen metabolism in premenopausal women presumed to be at high or low risk of breast cancer (34). They found that women at 'high risk' of breast cancer (family history of breast cancer or epithelial atypia in a previous biopsy) had a significantly higher (22% higher) extent of 16α-hydroxylation than women without high risk lesions or a family history ('low-risk' controls). High risk women had a similar elevated extent of 16α-hydroxylation of E2 as the breast cancer patients in the study described above (32). Translated to relative risks, Osborne et al.'s data suggest that one standard deviation increase in the extent of 16α-hydroxylation from the level of low risk controls may result in a 3-fold elevation of breast cancer risk. No data on total estrogen values, dietary or non-dietary risk factors were provided. No other studies have been reported attempting to confirm or refute the finding of Osborne et al. (34).

The hypothesis we are testing is whether postmenopausal women with breast cancer metabolize a significantly higher amount of E1 through 16α- than 2-hydroxylation compared to postmenopausal controls, independent of total urinary E1, E2 and E3. We expect the ratios of 16 α-hydroxy-metabolites to 2-hydroxy-metabolites to be statistically significantly higher in cases than in controls.

(6) Body:

METHODS

The methods we are using to obtain the sample are as follows:

Case selection: 100 cases will be selected using the criteria defined below:

Case selection- Eligibility Criteria:

1. Incident cases of female breast cancer identified through the Los Angeles County Cancer Surveillance Program (CSP; an NCI SEER registry), aged 55-64 years at time of diagnosis of histologically confirmed breast cancer.
2. Cancer ≤ stage II [tumor size $\leq T_2$, nodes $\leq N_1$, no distant metastasis (M_0), or T_3, N_0, M_0] (40).
3. A participant in an ongoing breast cancer case-control study at our institution (P.I. R. Ross, NIH 5 P01 CA 17054).

4. English speaking, Black or White (including Hispanic), resident in Los Angeles County at time of the case's diagnosis.
5. Over the past 6 months: not used medications that may interfere with estrogen metabolism (cimetidine, thyroxine, estrogen, progesterone, tamoxifen, or omega-3 fatty acid supplements).
6. Over the past 3 months: not have had general anesthesia.

Control selection:

We will enroll 100 controls from the non-cancer control group used for the breast cancer case-control study who satisfy eligibility criteria 3-9 above. (No further matching on age is necessary, since all participants are between 55-64 years of age). There will be no matching on weight, but we will adjust for weight in the statistical analysis of the results.

Recruitment procedures:

The research scientist will contact cases and controls who were most recently interviewed for the ongoing study, and then subsequently systematically contact women who participated previously. Cases and controls will be contacted strictly in order of recency of interview. Cases and controls who have moved are attempted to be traced through the Department of Motor Vehicle (DMV) files. Letters are sent to new addresses when these are obtained. Cases and controls are asked to provide a 60 ml sample of first void early morning urine. Thus, the first 100 cases and the first 100 controls identified from this process who satisfy the eligibility criteria and are willing to sign an informed consent are included.

A box containing a 100 ml urine vial with a 100 mg ascorbate tablet, a small cooler with multiple ice packs (only leaving room for the urine sample), an informed consent form and a questionnaire on recent intake of medication, alcohol and specific foods are shipped to each eligible woman who agrees to participate. The participants will be asked to place the urine sample in the cooler with the ice packs (previously frozen by the participant) immediately after it has been produced, and to enclose a signed informed consent form and the completed questionnaire on alcohol intake and current medication with the cooler.

For approximately half the participants the cooler with the urine has been picked up (by noon) the same day the urine sample is produced. For the other half of the participants the samples have been shipped with overnight express mail. The urine samples are divided into two samples of approximately 15 ml and immediately frozen at -70°C until shipped to the processing laboratories.

Dietary questionnaires and stamped return envelopes are sent to the participants after the urine has been received.

Batches of 30 samples, 15 from cases, 15 from controls are coded and shipped on dry ice via overnight express mail to Dr. Bradlow at the Strang-Cornell Cancer Research Laboratory, where the 16α-OHE1 and 2-OHE1 assays will be performed. At the same time as samples are shipped to Dr. Bradlow, we will also ship samples to Dr. Stanczyk at Los Angeles County/USC Women's Hospital, who will perform the E1, E2 and E3 assays. The only identifiers on the samples are code numbers ensuring that the laboratories will be blinded as to case or control status of the individual samples.

LABORATORY METHODS

The 16a-OHE1 and 2-OHE1 assays will be performed by Dr. H. Leon Bradlow at Strang-Cornell Cancer Research Laboratory in New York. The E1, E2 and E3 assays will be performed by Dr. Frank Stanczyk at the Reproductive Endocrinology Laboratory at the Los Angeles County/USC Women's Hospital.

Enzyme Immunoassay (EIA) of 16a-OHE1 and 2-OHE1:

This method has been described and validated by Klug et al. (41). In short, the EIAs used for these assays (Immuna Care Corporation, Bethlehem, PA) are competitive, solid-phase immunoassays. In this assay format, the antibody is immobilized on the solid phase and the antigen (estrogen metabolite) is labeled with the enzyme. In the test, binding of the antigen-enzyme conjugate by the immobilized antibody is inhibited by the addition of free antigen. Since a restricted number of antibody binding sites are available, the enzyme activity bound to the solid phase in the presence of free antigen is lowered. When enzyme substrate is added to the washed solid phase, the enzyme product (e.g., colored dye) concentration is inversely proportional to the concentration of the free antigen. In the current assay kits, monoclonal antibodies to estrogen metabolites are immobilized directly to the solid phase (wells of 8 x 12 polystyrene microtiter phase). The estrogen metabolites have been conjugated to alkaline phosphatase enzyme (AP).

The estrogens are deconjugated of both glucuronic acid and sulphate by use of a mixture of b-glucuronidase and arylsulphatase enzyme isolated from the snail Helix Pomatia. An aliquot of urine is diluted 1:20 with an acid buffer containing the enzymes and incubated until deconjugation is complete. The enzyme digest is then neutralized and used directly in the assay.

The intraassay (within assay) coefficients of variation (CVs) and the interassay (between-assay) CV for the two assays are all between 5-10%.

The EIA kits for urinary 16a-OHE1 and 2-OHE1 have been validated by comparing values obtained with these kits to values obtained by Gas Chromatography-Mass Spectroscopy (42). The correlation coefficient between the two methods was found to be 0.80 for both 16a-OHE1 and 2-OHE1.

Radioimmunoassay of Urinary E1, E2 and E3:

Urine (1 ml) is acidified with 2M acetate buffer (pH 5) and a mixture of β-glucuronidase/ arylsulfatase is added to hydrolyze estrogen conjugates. Deconjugation is carried out during a 24 hour incubation period at 37°C. Following the addition of approximately 1000 d.p.m. of ^{3}H-E1,^{3}H-E2, and ^{3}H-E3, which serve as internal standards to follow procedural losses, a selective extraction of E1, and E2, is carried out using diethyl ether (43). E3, which remains in the aqueous layer, is removed by extraction with 40% ethyl acetate in hexane.

The estrogens in the crude extracts are chromatographed using different solvent systems. E1 and E2 are applied on a column of Celite impregnated with ethylene glycol. E1 is eluted in 3.5 ml of 15% ethyl acetate in isooctane, and E2 in 5 ml of 50% ethyl acetate in isooctane. Similarly, the ethyl acetate/isooctane mixture containing E3 is applied on a column of Celite (impregnated with methanol: water (60:40::v:v). E3 is eluted with 30% ethyl acetate in isooctane.

The E1, E2 and E3 fractions are quantified by radioimmunoassay (RIA) using methods previously described by Stanczyk and colleagues (43-45). Separation of the antibody-bound and unbound estrogens is accomplished by either dextran-coated charcoal or double antibody techniques, employing standard procedures. The estrogen RIAs have been validated as described

previously (43-45). Appropriate quality controls are used with each set of samples that is assayed to monitor assay reliability. The intraassay and interassay CVs of each estrogen RIA are 5-10% and 10-15%, respectively.

Dietary Assessment:

Dietary factors may influence the extent of 16α- and 2-hydroxylation (46-48). We are therefore collecting data on dietary intake (including alcohol) over the past year using the dietary questionnaire developed by Dr. Willett and coworkers (49-50). In addition, information on medication and alcohol intake over the previous 48 hours as well as alcohol intake over the past month will be collected. We will also ask about the consumption of foods high in indole-3-carbinol over the past 48 hours.

STATISTICAL ANALYSIS

Results will be analyzed statistically using t-tests, standard analyses of covariance techniques, as well as logistic regression (51-54) using the statistical software packages SAS (SAS Institute Inc., Cary, NC) and EPILOG (Epicenter Software, Pasadena, CA). In the logistic regression, the odds ratio per unit increase in 16α–OHE1/2–OHE1, (16α–OHE1 + E3)/2–OHE1 and E3/2–OHE1 (with and without adjustment for urinary E1, E2 and E3), will be calculated. Other variables that will be considered as possible confounders are: age, race, body mass index, age at first birth, parity, age at and type of menopause, and dietary intake (such as total fat, protein, carbohydrate and alcohol intake).

RESULTS

We have so far contacted 407 cases and 445 controls. Responses have been obtained from approximately 700 women so far. Approximately 170 subjects (20%) have been found to be eligible so far. Major reasons for ineligibility are tamoxifen use (35% of cases) and estrogen use (38% of controls). Other reasons for ineligibility include: use of other medications (chemotherapy, thyroid medication etc.) (20%), moved, and not traceable through DMV (10%), refusal (5%), deceased (5% of cases), other (weight, recent surgery etc.) 10%. Currently, urine samples have been collected from 157 subjects (71 cases and 86 controls). This represents approximately 80% of the samples we said we would collect. The number of ineligible women, and the number of women who had moved and who therefore had to be traced through the DMV was higher than expected. There are some women who we have just recently localized through the DMV, and who have therefore only been contacted once. We have now just started recontacting these women. We are also in the process of identifying all other nonresponders and will contact them shortly. This should increase our number of eligible women to 200.

The first batches of urine samples have been sent to Dr. Bradlow in New York and Dr. Stanczyk at USC for analysis. Preliminary results of urine samples from the first 55 subjects did not yield significant differences between cases and controls on 16α-/2-OHE1. (The differences between cases and controls on E1, E2, E3 or the combination of the three were not quite statistically significant). However, because our study coincided with a reproducibility/validity study of the EIA assays of 16α- and 2-OHE1 conducted by Dr. Regina Ziegler, NCI, we decided earlier this spring to wait with our urinary analyses until the results from Dr. Ziegler's study were

available. Dr. Ziegler and coworkers found that the reproducibility of the assays of 16α- and 2-OHE1 in postmenopausal women was rather low (Regina Ziegler, NCI, unpublished data). As a result of this, the 16α- and 2-OHE1 assays have during the past few months undergone adjustments to account for the lower levels of estrogens in urine of postmenopausal women (Leon Bradlow, personal communication). The new, adjusted assays are currently being validated by Ziegler and colleagues at NCI. We have therefore decided to wait with sending further samples until the results of Dr. Ziegler's study suggests that the reproducibility of the assays has been improved. We expect these problems to be resolved within a few months. Because of these reproducibility problems of the EIAs for 16α-OHE1 and 2-OHE1, we requested (and have obtained) a 1-year no-cost extension of this grant.

The questionnaires are checked for inconsistencies as they are obtained. The dietary questionnaires are normally shipped in batches of 100 to Harvard. As we are collecting data on 200 women, we have therefore decided to wait with sending these to Harvard until all the questionnaires have been collected. The other risk factor information obtained for this study is being prepared for key-punching. The data will be key-punched all at one time when all the urine samples have been collected.

(7) Conclusions

Because of the reproducibility problems with the 16α- and 2-OHE1 assays discussed above, we would prefer to not draw any implications from our results so far. It is also too early to suggest changes for future projects, except perhaps that it would be useful to request funds for a separate validity/reproducibility study if a new method is being used.

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