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The molecular etiology of breast cancer: Evidence from biomarkers of risk

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Abstract

Estrogens can become endogenous carcinogens *via* formation of catechol estrogen quinones, which react with DNA to form specific depurinating estrogen-DNA adducts. The mutations resulting from these adducts can lead to cell transformation and the initiation of breast cancer. Estrogen metabolites, conjugates and depurinating DNA adducts in urine samples from 46 healthy control women, 12 high-risk women and 17 women with breast cancer were analyzed. The estrogen metabolites, conjugates and depurinating DNA adducts were identified and quantified by using ultra-performance liquid chromatography/tandem mass spectrometry. The levels of the ratios of depurinating DNA adducts to their respective estrogen metabolites and conjugates were significantly higher in high-risk women ($p < 0.001$) and women with breast cancer ($p < 0.001$) than in control subjects. The high-risk and breast cancer groups were not significantly different ($p = 0.62$). After adjusting for patient characteristics, these ratios were still significantly associated with health status. Thus, the depurinating estrogen-DNA adducts are possible biomarkers for early detection of breast cancer risk and response to preventive treatment.

Keywords

breast cancer risk; depurinating estrogen-DNA adducts; estrogen biomarkers; balance in estrogen metabolism

Development of noninvasive tests of breast cancer risk has been a major goal for more than 30 years. In this article we present bio-markers of risk that are related to the hypothesized first critical step in the initiation of breast cancer, namely, the reaction of catechol estrogen

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quinone metabolites with DNA.[1] Prevention of cancer can be achieved by blocking this DNA damage, which generates the mutations leading to the initiation, promotion and progression of cancer.[2]

Exposure to estrogens is a known risk factor for breast cancer.[3,4] The discovery that specific oxidative metabolites of estrogens, namely, catechol estrogen quinones, can react with DNA[5–9] led to and supports the hypothesis that these metabolites can become endogenous chemical carcinogens. Some of the mutations generated by this specific DNA damage can result in the initiation of cancer.[1,5] This paradigm suggests that specific, critical mutations generate abnormal cell proliferation leading to cancer.[1,10–13]

As illustrated in Figure 1, in the metabolism of catechol estrogens there are activating pathways[14] that lead to the formation of the estrogen quinones, estrone (estradiol) quinones [$E_1(E_2)$-Q], which can react with DNA. There are also deactivating pathways that limit formation of the quinones and/or prevent their reaction with DNA. These are methylation of catechol estrogens,[15] conjugation of the $E_1(E_2)$-Q with glutathione (GSH)[16] and reduction of the quinones to catechols[17] (Fig. 1).

When $E_1(E_2)$-3,4-Q react with DNA, they form predominantly the depurinating adducts 4-hydroxyestrone(estradiol)-1-N3Ade-nine [4-$OHE_1(E_2)$-1-N3Ade] and 4-hydroxyestrone(estradiol)-1-N7Guanine [4-$OHE_1(E_2)$-1-N7Gua],[5–7] whereas $E_1(E_2)$-2,3-Q form much lower levels of 2-hydroxyestrone(estradiol)-6-N3Ade-nine [2-$OHE_1(E_2)$-6-N3Ade] (Figs. 1 and 2).[7] Both $E_1(E_2)$-3,4-Q and $E_1(E_2)$-2,3-Q form much lower levels of stable DNA adducts than depurinating adducts.[5–7] Once released from the DNA, the depurinating estrogen-DNA adducts are shed from cells into the bloodstream and, eventually, are excreted in urine.

The release of the depurinating adducts generates apurinic sites in DNA, which in turn, may induce mutations. The observation of Harvey-*ras* mutations within 6–12 hr after treatment of mouse skin or rat mammary glands with E_2-3,4-Q suggests that these mutations arise *via* error-prone base excision repair.[1,10,11] Similar patterns of mutations have also been observed in the big blue (BB) rat mammary gland and cultured BB rat2 embryonic cells after treatment with 4-hydroxyestradiol (4-OHE_2) or E_2-3,4-Q.[1,12] The transforming activity of E_2 and 4-OHE_2 has been observed in human breast epithelial (MCF-10F) cells, which do not contain estrogen receptor-α, and it is not affected by the presence of an anti-estrogen.[18–20] Furthermore, 4-$OHE_1(E_2)$ are carcinogenic in the Syrian golden hamster and CD-1 mouse.[21–24] All of these results support the hypothesis that estrogens initiate cancer through their genotoxicity.

Initiation of cancer by estrogens is based on estrogen metabolism in which the homeostatic balance between activating and deactivating pathways is disrupted (Fig. 1). Activating pathways are the ones that oxidize E_1 and E_2 to their catechol estrogen quinones, whereas the deactivating pathways are the ones that block oxidation.[1] A variety of factors, such as diet, environment and lifestyle, can unbalance the equilibrium between these 2 pathways. When estrogen metabolism is balanced, the level of estrogen-DNA adducts in tissue and urine is low and/or the levels of estrogen metabolites and conjugates are high. In contrast,

when estrogen metabolism is unbalanced, the level of DNA adducts in tissue and urine is high and/or the levels of estrogen metabolites and conjugates are low. It is this imbalance in estrogen metabolism, leading to relatively high levels of estrogen-DNA adducts, that may be a critical determinant of breast cancer initiation.

The above considerations led us to hypothesize that estrogen metabolites, conjugates and depurinating DNA adducts may differ between healthy women and women with breast cancer or at high risk of breast cancer. To test this hypothesis, we conducted a cross-sectional study in which 40 estrogen metabolites, conjugates and depurinating DNA adducts were analyzed in urine samples from healthy women, women at high risk for breast cancer based on Gail Model score >1.66%, and women with breast carcinoma. The Gail Model takes into account the following factors: age, age at menarche, age at first live birth, number of breast biopsies and history of atypical hyperplasia, number of first degree relatives with breast cancer (mother, sister and daughter) and race. A 5-year Gail Model score of >1.66% is considered high risk.[25]

Material and methods

Materials

Phenyl solid phase extraction (SPE) cartridges were purchased from Varian (Palo Alto, CA). Androstenedione (**1**), (Table I), testosterone (**2**), estrone (E_1) sulfate (**3**), E_2 (**4**), E_1 (**5**), 2-OHE_2 (**6**), 2-OHE_1 (**7**), 16α-OHE_2 (**10**), 16α-OHE_1 (**11**), 2-OCH_3E_2 (**12**), 2-OCH_3E_1 (**13**), 4-OCH_3E_2 (**14**), 4-OCH_3E_1 (**15**), 2-OH-3-OCH_3E_2 (**16**) and 2-OH-3-OCH_3E_1 (**17**) were purchased from Steraloids (Newport, RI). 4-OHE_2 (**8**) and 4-OHE_1 (**9**) were synthesized as previously described.[26] 2-OHE_2-1-SG (**18**), 2-OHE_2-4-SG (**19**), 2-OHE_1-1-SG (**20**), 2-OHE_1-4-SG (**21**), 2-OHE_2-(1+4)-Cys (**22**), 2-OHE_1-1-Cys (**23**), 2-OHE_1-4-Cys (**24**), 2-OHE_2-1-NAcCys (**25**), 2-OHE_2-4-NAcCys (**26**), 2-OHE_1-1-NAcCys (**27**), 2-OHE_1-4-NAcCys (**28**), 4-OHE_2-2-SG (**29**), 4-OHE_1-2-SG (**30**), 4-OHE_2-2-Cys (**31**), 4-OHE_1-2-Cys (**32**), 4-OHE_2-2-NAcCys (**33**) and 4-OHE_1-2-NAcCys (**34**) were synthesized by using the procedure of Cao *et al*.[27] 4-OHE_2-1-N7Gua (**35**), 4-OHE_1-1-N7Gua (**36**), 4-OHE_2-1-N3Ade (**37**), 4-OHE_1-1-N3Ade (**38**), 2-OHE_2-6-N3Ade (**39**) and 2-OHE_1-6-N3Ade (**40**) were synthesized by following the reported methods.[6,7,28] All solvents were HPLC grade and all other chemicals used were of the highest grade available.

Study population

We collected urine from 75 women at 3 different sites: (*i*) at the Center for Mammographic Screening at the University of Naples, Italy (42 women), (*ii*) at the Breast Diagnostic Clinic and Oncology Breast Clinic of the Mayo Clinic, Rochester, MN (18 women) and (*iii*) at the Olson Center for Women's Health, University of Nebraska Medical Center (UNMC), Omaha, NE (15 women). Women were recruited between March 2005 and September 2006 and their ages ranged between 34 and 73 years—healthy women: range, 34–67; mean, 50 ± 8; high-risk women: range, 44–64; mean, 52± 6; women with breast cancer: range, 34–73; mean, 54 ± 10.

All women recruited at the University of Naples were healthy (they did not receive a diagnosis of breast cancer at the time of their mammographic test). Among the women recruited at the Mayo Clinic, 12 were classified as high-risk women (Gail Model score = 1.67%–11.7%) and 6 were breast cancer cases. At UNMC, 4 women were healthy, that is, had no known cancer, and 11 were diagnosed with breast cancer. None of the subjects received estrogen-containing treatment for at least 3 months prior to providing a urine sample. The 3 groups were frequency matched on age, race and menopausal status.

All procedures were approved by the University of Naples, Mayo Clinic and UNMC Institutional Review Boards. Signed consents included authorization to collect and bank urine samples and collect demographic and clinical information.

Sample collection

A standardized method was followed to collect all of the urine samples. A spot urine sample of about 50 ml was collected from each participant and 1 mg/ml ascorbic acid was added to prevent oxidation of the catechol moieties in the various estrogen compounds. The urine samples were aliquoted, frozen and four 10-ml aliquots were transferred to the Eppley Institute, UNMC, on dry ice and were stored at −80°C until analysis. Thus, each analytical sample was thawed only once prior to analysis.

Solid-phase extraction of urine

Two milliliter aliquots of urine were partially purified by SPE. The SPE was performed using a 20-port SPE vacuum manifold with phenyl cartridges (Fig. 2). Urine samples were adjusted to pH 7 with 1 M NaOH or 1 M HCl. For method development and validation, 2-ml aliquots of charcoal-treated human urine samples were spiked with a total of 250, 500 or 1,000 pg of the 40 estrogen-related compounds (final concentration 0.125, 0.25 and 0.50 pg/μl) and loaded onto the phenyl 100-mg cartridges preconditioned with CH_3OH and the loading buffer, 10 mM ammonium formate, pH 7. The cartridges were washed with the loading buffer, and then the compounds of interest were eluted from the cartridge by using an elution buffer, methanol/10 mM ammonium formate, pH 7 (90:10) with 1% acetic acid. This procedure led to enrichment of the 40 estrogen-related compounds after elution. Charcoal-treated urine (2 ml) was used in controls, and the eluates from the SPE cartridges were spiked with 250, 500 or 1,000 pg of the 40 estrogen-related compounds. The eluates from both the experimental and control samples were concentrated using a Speed-Vac and lypholizer, and subjected to ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS-MS) analysis. To determine the recovery of the standards by the SPE method, comparison was made between the corresponding concentrations of experimental and control samples (Fig. 3). Study samples were cleaned in duplicate by using the above optimized SPE conditions and analyzed by UPLC/MS-MS.

UPLC/MS-MS analysis of urine samples

The 40 analytes (Table I) included the androgens androstenedione and testosterone; the estrogens E_1 sulfate, E_1 and E_2; the catechol estrogens 2-$OHE_1(E_2)$ and 4-$OHE_1(E_2)$; the 16α-$OHE_1(E_2)$; the methylated 2- and 4-catechol estrogens; the 2- and 4-catechol estrogens conjugated with GSH, cysteine (Cys) or *N*-acetylcys-teine (NAcCys); and the depurinating

DNA adducts of 4-$OHE_1(E_2)$ and 2-$OHE_1(E_2)$. All of the estrogen compounds were analyzed as both E_1 and E_2 derivatives because the interconversion of these 2 estrogens is carried out continuously by 17β-estradiol dehydrogenase.

All experiments were performed on a Waters (Milford, MA) Quattro Micro triple quadrupole mass spectrometer by using electrospray ionization (ESI) in positive ion (PI) and negative ion (NI) mode, with an ESI-MS capillary voltage of 3.0 kV, an extractor cone voltage of 2 V, and a detector voltage of 650 V. Desolvation gas flow was maintained at 600 l/h. Cone gas flow was set at 60 l/h. Desolvation temperature and source temperature were set to 200 and 100°C, respectively. For all the studies, a methanol:-water (1:1) mixture with 0.1% formic acid was used as the carrier solution. ESI interface tuning and mass calibration were accomplished in the PI mode by using a standard sodium iodide-rubidium iodide solution. The test sample (compounds 1 through 40) was introduced to the source at a flow rate of 10 μl/min by using an inbuilt pump. PI or NI detection was used in cases where the sample was readily ionized to cation or anion, respectively. The masses of parent ion and daughter ions were obtained in the MS and MS-MS operations. The parent and daughter ion data obtained for each compound were used to generate the multiple reaction monitoring (MRM) method for UPLC/MS-MS operation (Table I).

Measurements of estrogen-related compounds in urine extracts were conducted by using UPLC/MS-MS. UPLC/MS-MS analyses were carried out with a Waters Acquity UPLC system connected with the high-performance Quattro Micro triple quadrupole mass spectrometer. Analytical separations on the UPLC system were conducted using an Acquity UPLC BEH C18 1.7 μm column (1 × 100 mm) at a flow rate of 0.15 ml/min. The gradient started with 80% A (0.1% formic acid in H_2O) and 20% B (0.1% formic acid in CH_3CN), changed to 79% A over 4 min, followed by a 6-min linear gradient to 45% A, resulting in a total separation time of 10 min. The elutions from the UPLC column were introduced to the Quattro Micro mass spectrometer.

The ionization method used for MS analysis was ESI in both the PI and NI mode. MS-MS was performed in the MRM mode (see above), and resulting data were processed by using QuanLynx software (Waters) to quantify the estrogen metabolites. To calculate limits of detection, various concentrations, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50 and 100 pg/μl, of the analyte were injected to UPLC/MS-MS. The injected amount that resulted in a peak with a height at least 2 or 3 times as high as the baseline noise level was used as the limit of detection (Table I). Pure standards were used to optimize the UPLC/MS conditions prior to analysis. After UPLC analysis, the mean value was calculated for all the compounds obtained from each sample.

Statistical methods

Estrogen-related compounds were compared for control *versus* high risk and for control versus breast cancer using a Mann–Whitney test, with *p*-values adjusted for the 2 multiple comparisons using the Bonferroni method. To account for the multiple hypothesis tests conducted for these variables, a p-value <0.01 was interpreted as statistically significant. The log-transformed sum of the ratios of depurinating adducts to the corresponding metabolites and conjugates was compared using a one way ANOVA, and *post hoc*

comparisons were made using the method of Bonferroni. Linear regression was used to assess the association between disease status and ratio adjusted for age at recruitment, age at menarche, menopausal status (categorical) and parity for the 56 subjects with patient characteristics available. All the statistics and *p*-values were calculated using SPSS software (SPSS, Chicago, IL).

Results and discussion

Analysis of urine samples

After partial purification of the urine samples by SPE (Fig. 2), we analyzed the 40 estrogen-related compounds using UPLC/MS-MS. The advantage of having MS detector in MRM mode over conventional high pressure liquid chromatography analysis is that number of channels in the detector could be set to specifically and separately identify all the estrogen related compounds (Fig. 2). Each metabolite was detected and identified based on the parameters that are unique to them, such as mass (parent and daughter), retention time and ionization mode (positive and negative) (Table I). The typical spectra of representative estrogen derivatives, which were obtained in a single injection, are shown in Figure 2. The levels of estrogen-related compounds for a high risk woman, measured from single injection, are presented in Table II.

Treatment of urine with glucuronidase/sulfatase led to significant increases (10 to 20-fold) in the levels of E_1 and E_2, while the levels of estrogen metabolites, conjugates and adducts changed marginally and in many cases decreased because of the incubation for 8 hr at 37°C. To avoid artifacts and errors that are introduced by maintaining the urine samples at 37°C for 8 hr, we carried out all the analyses without glucuronidase/sulfatase treatment. Therefore, the observed levels of E_1 and E_2, as reported in Table II, for example, were 10 to 20-fold lower than the total values. Since estrone and estradiol are constantly inter-converting, we have combined estrone and estradiol values of all the derivatives (Tables II and III). The GSH conjugates of estrogen quinones are further converted to Cys and NAcCys conjugates *via* the mercapturic acid biosynthesis pathway.[29] Hence we have combined all the values of 2 conjugates and 4 conjugates (Tables II and III), which reflect the total protection by GSH from 2 or 4 quinones, respectively. The results presented here clearly demonstrate the ability of SPE combined with UPLC/MS-MS analysis to resolve, identify and quantify 40 estrogen-related compounds with accuracy and speed.

The values obtained for the various estrogen-related compounds in 3 groups of women were processed in 2 different ways. First, median values were calculated for all the compounds and their levels were examined in the 3 groups of women (Table III). Then, we used the ratio of depurinating N3Ade and N7Gua adducts to the sum of their respective estrogen metabolites and conjugates in urine samples because the ratio reflects the degree of imbalance in estrogen metabolism that can lead to cancer initiation (Fig. 4). A high ratio of adducts to their respective metabolites and conjugates represents relatively more DNA damage. In contrast, a low ratio of adducts to their respective metabolites and conjugates means that relatively little of the estrogen metabolites reacted with DNA.

Median values of the urinary estrogen-related compounds in the 3 groups of women

Using the newly developed SPE/UPLC/MS-MS methodology, we have analyzed urine samples of various women's groups for estrogen-related compounds. The data obtained were used to calculate median values for each of the 40 compounds (Table III).

The median andostenedione, testosterone, E_2/E_1, 16α-OHE_2/ 16α-OHE_1, 4-OCH_3E_2/4-OCH_3E_1, 2-$OHE_1(E_2)$ GSH conjugate and derivative values were higher for controls compared to high risk participants, and the median 4-OHE_2-1-N3Ade/4-OHE_1-1-N3Ade values were lower for controls compared to high risk participants. Compared to breast cancer participants, the median 2-OHE_2/2-OHE_1, 4-OHE_2/4-OHE_1, 16α-OHE_2/16α-OHE_1, 4-OCH_3E_2/4-OCH_3E_1, 2-$OHE_1(E_2)$ GSH conjugate and derivative values were higher for controls, while the median 4-OHE_2-1-N7Gua/4-OHE_1-1-N7Gua values were lower for controls. Of particular interest are the significantly lower levels of the methoxycatechol estrogens in the women with breast cancer or at high risk compared to the control women, because this represents a major protective pathway in estrogen metabolism. In addition, the levels of the 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts are higher in the women with breast cancer or at high risk than in the control women, although only 2 of the differences are statistically significant.

Depurinating estrogen-DNA adducts in the 3 groups of women

In the second analysis, the ratios of depurinating N3Ade and N7Gua adducts to the sum of estrogen metabolites and conjugates in urine samples from healthy control women are generally low (Fig. 4). In contrast, high ratios of these adducts to estrogen metabolites and conjugates were observed in urine from high-risk women (Gail Model score >1.66%) and women with breast carcinoma. In general, the value obtained from the high-risk women and women with breast carcinoma derives from the ratio between a high level of adducts and low levels of metabolites and conjugates. In some women, however, the level of adducts was not particularly high, but the levels of metabolites and conjugates were very low, suggesting that a substantial proportion of the metabolites was converted to adducts.

In the sum of the ratios of depurinating adducts to estrogen metabolites and conjugates, the preponderant role is played by the N3Ade and N7Gua adducts of 4-$OHE_1(E_2)$, whereas the adducts of 2-$OHE_1(E_2)$ play a very minor role. For example, for the high-risk subject presented in Table II, the overall adduct ratio is 936, but the contribution of 2-$OHE_1(E_2)$-6-N3Ade is 1, whereas the contribution of 4-$OHE_1(E_2)$-1-N3Ade plus 4-$OHE_1(E_2)$-1-N7Gua is 935. In general, the average contribution of the 2-$OHE_1(E_2)$-6-N3Ade adducts is ~2.5% of the total, whereas the predominant contribution of ~97.5% derives from the 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts. The observation of high levels of depurinating estrogen-DNA adducts in urine from high-risk women, as well as subjects with breast carcinoma (Fig. 4), is consistent with the hypothesis that these adducts are a causative factor in the etiology of breast cancer.

Analysis by subject characteristics

We first analyzed the data using the ratio of depurinating N3Ade and N7Gua adducts to the sum of their respective estrogen metabolites and conjugates in urine samples as a continuous

variable. Analysis using one-way ANOVA revealed a significant difference among the groups ($p < 0.001$). Additional *post hoc* analysis using a Bonferroni correction for multiple comparisons revealed significantly higher means for high risk subjects [mean 336.45, standard deviation (SD) 331.92] compared to controls (mean 20.51, SD 37.01, $p < 0.001$) and for breast cancer patients (mean 176.28, SD 205.68, $p < 0.001$). The mean for patients known to be at high risk was not significantly different from that of the breast cancer group ($p = 0.62$).

A limitation of the study is that most of the group of healthy women (42 of 46) were Italian, whereas the remaining healthy women, high-risk women and women with breast cancer were American. All of the subjects in our study, however, were Caucasian. The 3 groups (healthy, high-risk and breast cancer) had similar mean age at recruitment, mean age at menarche and menopausal status (Table IV). These similarities in subject characteristics support the validity of comparing the ratios of adducts to their respective metabolites and conjugates in these 3 groups of women.

Subject characteristics of age at recruitment, age at menarche, menopausal status, and parity were available for 56 of the 75 subjects (Table IV). The mean age of our entirely Caucasian sample was 50 years (SD 8.5). The average age at menarche was 12.0 years (SD 1.4). Only 11% of the women were nulliparous and 43% had at least 2 children. Twenty-six (46%) women were pre-menopausal at recruitment, 30 (54%) were postmenopausal (they did not have menstrual cycles in the last 12 months before recruitment). Analysis using one way ANOVA revealed that health status, that is breast cancer cases *versus* high risk and healthy individuals, was significantly associated with age at recruitment ($p = 0.048$). Specifically, the mean age (years) at recruitment for healthy women was 49 (SD 7.8), 52 (SD 6.1) for women at high risk and 57 (SD 12.2) for breast cancer cases. Age at menarche was not statistically different across the disease status groups ($p = 0.534$). Analysis using a χ^2 test did not reveal an association between health status and menopausal status ($p = 0.95$) or parity (parous *vs*. nulliparous) ($p = 0.15$).

The correlation coefficient was used to examine the association between the ratio and subject characteristics. We observed evidence of significant correlation between parity and ratio ($r = 0.36$, $p = 0.007$) and marginally significant correlation between the ratio and menopausal status ($r = 0.26$, $p = 0.06$). Age at recruitment and age at menarche were not significantly associated with the ratio.

Linear regression was used to assess the association between disease status and ratio adjusted for age at recruitment, age at menarche, menopausal status (categorical) and parity for the 56 subjects with patient characteristics available (Table V). After accounting for these characteristics, the ratio was significantly associated with health status. Specifically, the multivariate coefficient for disease status (108.6) was statistically significant ($p = 0.007$) in a model that explained 10% ($p = 0.040$) of variance in the ratio after accounting for covariates. All other covariates did not reach the usual level of significance of 0.05 (Table V).

Interpretation of results

The observation of high ratios of depurinating estrogen-DNA adducts to their corresponding metabolites and conjugates in urine samples from both high-risk women and women with breast cancer supports the hypothesis that formation of estrogen-DNA adducts is the first critical step in the initiation of breast cancer.[1] In addition, these results suggest that this assay may provide a diagnostic tool for early detection of breast cancer risk. At this point, we do not know how far in advance this assay would predict the development of a detectable tumor. Further studies are required to address this question.

In addition, we can hypothesize that the ratio of depurinating estrogen-DNA adducts to their metabolites and conjugates can be used to monitor the efficacy of putative preventive compounds in balancing estrogen activation and deactivation. Minimizing formation of catechol estrogen quinones and/or their reaction with DNA should reduce the risk of developing breast cancer.

Conclusions

UPLC/MS-MS can be used to analyze depurinating estrogen-DNA adducts, estrogen metabolites and estrogen conjugates in 2-ml urine specimens. The ratio of adducts to their corresponding metabolites and conjugates provides a biomarker that can be used to distinguish women known to be at high risk of developing breast cancer (Gail Model score >1.66%) and those with breast cancer from healthy control women. The development of such bio-markers could be invaluable in assessing breast cancer risk and response to preventive treatment.

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Abbreviations

Cys	cysteine
ESI	electrospray ionization
$E_1(E_2)$-Q	estrone(estradiol)-quinones
GSH	glutathione
4-OHE_2	4-hydroxyestra-diol
4-$OHE_1(E_2)$-1-N3Ade	4-hydroxyestrone(estradiol)-1-N3Adenine
4-$OHE_1(E_2)$-1-N7Gua	4-hydroxyestrone(estradiol)-1-N7Guanine
2-$OHE_1(E_2)$-6-N3Ade	2-hydroxyestrone(estradiol)-6-N3Adenine

MRM	multiple reaction monitoring
NAcCys	*N*-acetylcysteine
NI	negative ion
PI	positive ion
SPE	solid-phase extraction
UPLC/MS-MS	ultraperformance liquid chromatography/tandem mass spectrometry

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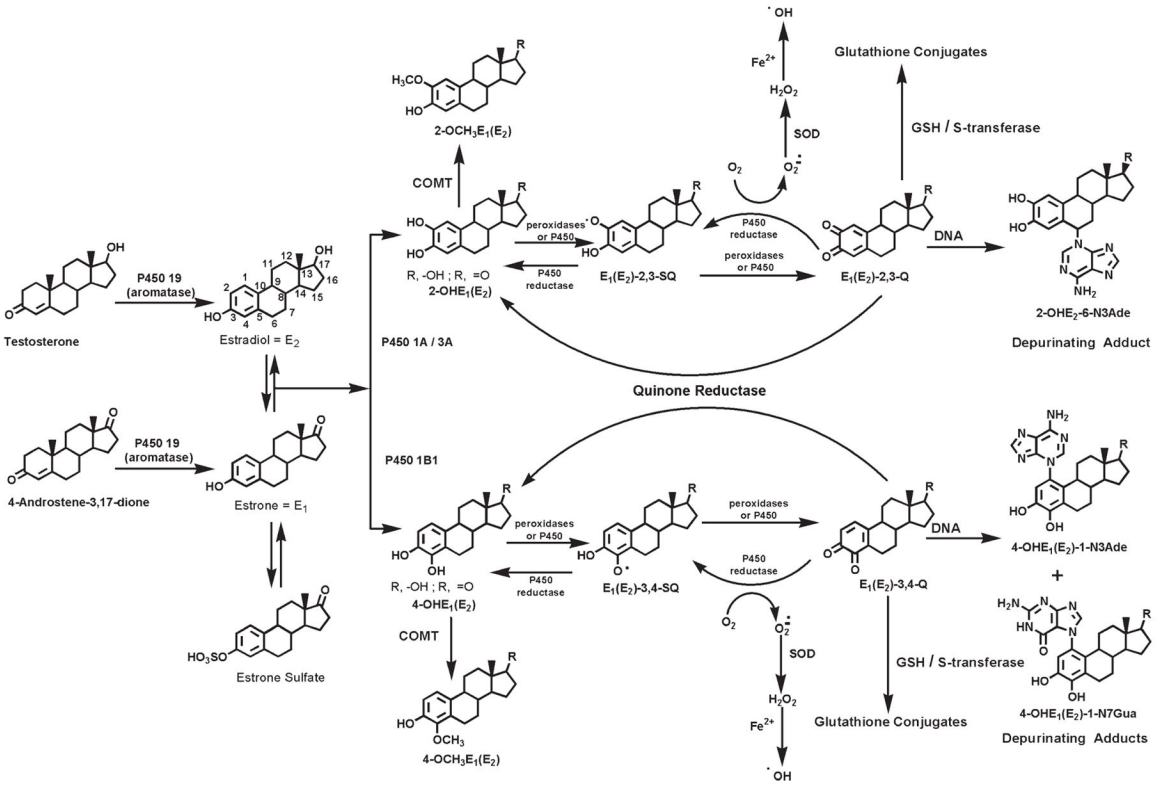

FIGURE 1.

Biosynthesis and metabolic activation of the estrogens, E_1 and E_2. The metabolic activation of E_1 and E_2 leads to 2- and 4-catechol derivatives, which further oxidize to yield the corresponding reactive quinones. The quinones react with DNA to form depurinating DNA adducts. In the deactivation pathway, which operates in parallel, the catechol derivatives are methylated to form methoxy catechol estrogens; in addition, the quinones are reduced by quinone reductase, as well as are conjugated with GSH, and, thus, are rendered harmless. The shift in the apparent balance between these activating and deactivating pathways towards formation of depurinating DNA adducts could lead to the initiation of breast cancer.

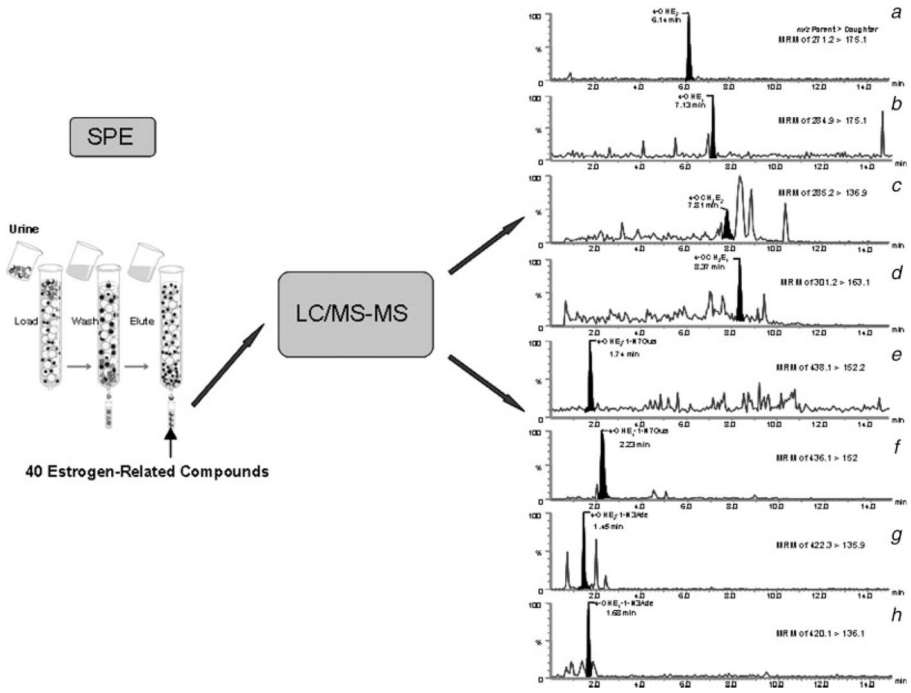

FIGURE 2.

Schematic representation of the steps carried out to purify by SPE and analyze by UPLC/MS-MS the estrogen-related compounds from urine samples. The UPLC/MS-MS chromatograms of (*a*) 4-OHE_2, (*b*) 4-OHE_1, (*c*) 4-OCH_3E_2, (*d*) 4-OCH_3E_1, (*e*) 4-OHE_2-1-N7Gua, (*f*) 4-OHE_1-1-N7Gua, (*g*) 4-OHE_2-1-N3Ade and (*h*) 4-OHE_1-1-N3Ade that are shown in the figure are representatives from the 40 different estrogen-related compounds seen in the urine samples.

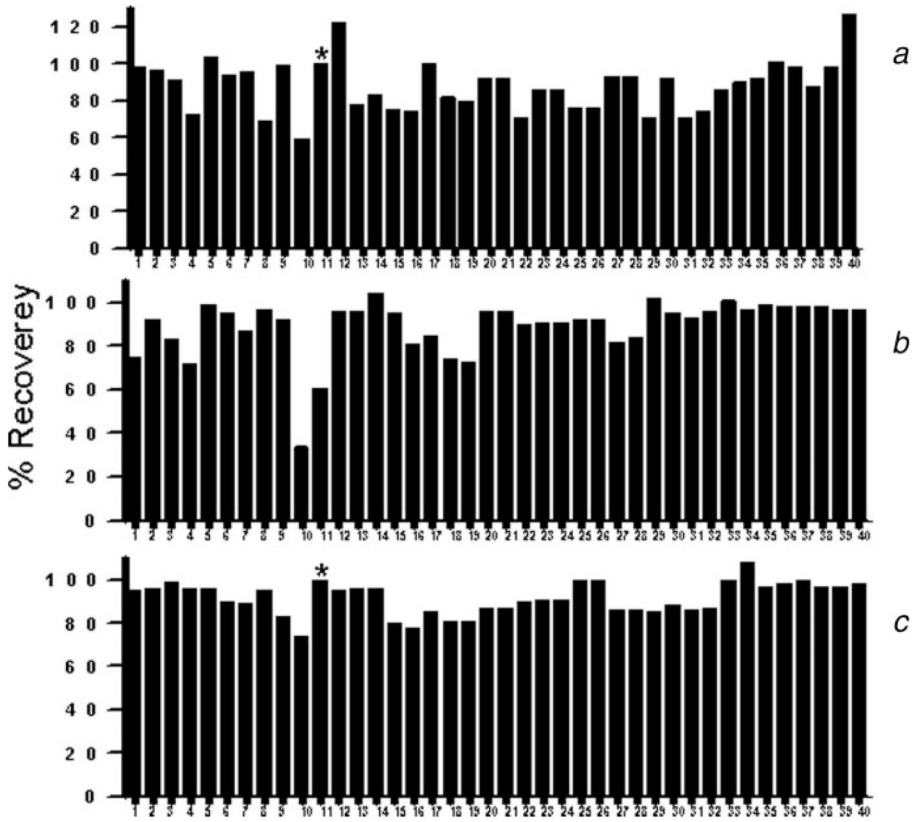

FIGURE 3.

SPE recovery of standard 40 estrogen-related compounds. The 2-ml aliquots of activated charcoal-treated human urine samples were spiked with the total (*a*) 250, (*b*) 500 and (*c*) 1,000 pg of 40 estrogen-related compounds before and after (control) passing over phenyl SPE cartridges. The recovery of each compound was determined by comparing the experimental values to the controls.

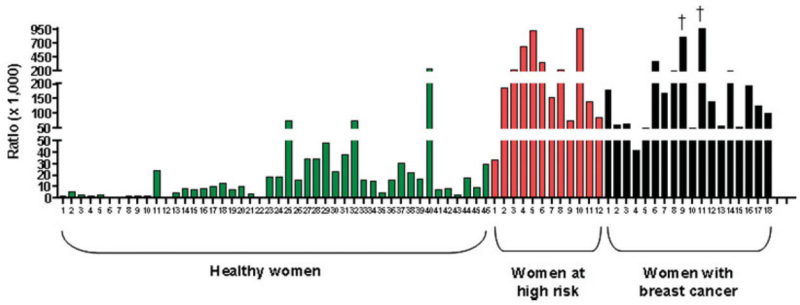

FIGURE 4.

Depurinating estrogen-DNA adducts in the urine of healthy women, high-risk women and women with breast cancer. The ordinate of this bar graph corresponds to the ratio of depurinating DNA adducts divided by their respective estrogen metabolites and conjugates:

$$\left(\frac{4\text{-OHE}_1(\text{E}_2)\text{-1-N3Ade}+4\text{-OHE}_1(\text{E2})\text{-1-N7Gua}}{4\text{-catechol estrogens}+4\text{-catechol estrogen conjugates}}+\frac{2\text{-OHE}_1(\text{E}_2)\text{-6-N3Ade}}{2\text{-catechol estrogens}+2\text{-catechol estrogen conjugates}}\right)\times 1000$$

The mean sum of the ratios for control women was significantly lower than those for the high-risk women ($p < 0.001$) and women with breast cancer ($p < 0.001$). The mean sums of the ratios for high-risk women and women with breast cancer were not significantly different ($p = 0.62$). †These are 2 urine samples from the same subject, collected 11 weeks apart. Statistical calculations used 1 average value for this subject.

TABLE I

MASS SPECTROMETRIC PARAMETERS[1]

No.	Compound	Mass	ESI mode	Parent (*m/z*)	Daughters (*m/z*)	Cone (volt)	Collision	Retention time	LOD (fmol)
1	Androstenedione	286.2	Positive	287.1	97.1	40	19	8.43	14
2	Testosterone	288.2	Positive	289.2	97.0	40	19	7.97	35
3	E_1-Sulfate	350.1	Negative	249.0	269.0	50	28	6.61	143
4	E_2	272.4	Positive	273.2	135.2	30	14	7.74	184
5	E_1	270.1	Positive	271.2	253.2	25	14	8.43	148
6	2-OHE_2	288.2	Positive	271.2	175.1	30	14	6.74	69
7	2-OHE_1	286.2	Negative	285.0	160.9	65	37	7.3	18
8	4-OHE_2	288.2	Positive	271.2	175.1	30	14	6.14	69
9	4-OHE_1	286.2	Negative	284.9	161.0, 175.1	65	35	7.13	35
10	16α-OHE_2	288.4	Positive	289.0	107.0	25	14	2.42	867
11	16α-OHE_1	286.4	Negative	285.1	145.1	30	15	4.67	349
12	2-OCH_3E_2	302.2	Positive	285.2	136.9, 189.1	32	15	8.25	330
13	2-OCH_3E_1	300.2	Positive	301.2	136.9, 163.1	30	17	8.85	333
14	4-OCH_3E_2	302.2	Positive	285.2	136.9, 189.1	32	15	7.81	66
15	4-OCH_3E_1	300.2	Positive	301.2	163.1, 283.1	30	17	8.37	133
16	2-OH-3-OCH_3E_2	302.4	Positive	285.2	189.1	32	15	8.71	165
17	2-OH-3-OCH_3E_1	300.4	Positive	301.2	163.1	30	17	9.07	33
18	2-OHE_2-1-SG	593.7	Positive	594.1	319.1, 465.0	42	20	1.72	8.4
19	2-OHE_2-4-SG	593.7	Positive	594.0	319.1, 465.4	35	21	2.32	8.4
20	2-OHE_1-1-SG	591.0	Positive	592.1	316.8	45	22	2.65	1.7
21	2-OHE_1-4-SG	591.0	Positive	592.2	317.1, 463.2	40	22	2.65	1.7
22	2-OHE_2-1+4-Cys	407.2	Positive	408.2	319.0	30	17	1.73	12
23	2-OHE_1-1-Cys	405.2	Positive	406.0	316.9	35	15	3.25	6.2
24	2-OHE_1-4-Cys	405.2	Positive	406.2	317.1	30	17	3.25	6.2
25	2-OHE_2-1-NAcCys	449.2	Positive	450.1	162.0, 287.4	25	14	4.07	5.6
26	2-OHE_2-4-NAcCys	449.2	Positive	450.2	162.0, 287.2	30	14	4.07	5.6

No.	Compound	Mass	ESI mode	Parent (*m/z*)	Daughters (*m/z*)	Cone (volt)	Collision	Retention time	LOD (fmol)
27	2-OHE_1-1-NAcCys	447.2	Positive	448.1	162.0, 285.4	30	13	6.05	5.6
28	2-OHE_1-4-NAcCys	447.2	Positive	448.0	162.0, 284.9	35	14	6.05	5.6
29	4-OHE_2-2-SG	593.2	Positive	594.4	318.9, 464.8	42	20	2.33	8.4
30	4-OHE_1-2-SG	591.2	Positive	592.3	317.1, 462.9	45	22	2.65	8.5
31	4-OHE_2-2-Cys	407.2	Positive	408.0	318.9, 286.1	40	16	2.24	2.4
32	4-OHE_1-2-Cys	405.2	Positive	406.0	316.9, 389.0	35	15	2.84	6.2
33	4-OHE_2-2-NAcCys	449.2	Positive	450.1	162.1	35	15	5.91	5.6
34	4-OHE_1-2-NAcCys	447.2	Positive	448.3	161.8	35	14	6.64	2.2
35	4-OHE_2-1-N7Gua	437.2	Positive	438.1	152.2, 272.0	62	38	1.74	2.3
36	4-OHE_1-1-N7Gua	435.2	Positive	436.1	152.0, 271.9	62	38	2.23	2.2
37	4-OHE_2-1-N3Ade	421.2	Positive	422.3	135.9, 257.1	62	45	1.45	5.9
38	4-OHE_1-1-N3Ade	419.2	Positive	420.1	296.0, 136.1	60	44	1.68	2.4
39	2-OHE_2-6-N3Ade	421.1	Positive	422.2	136.0, 287.0	26	10	1.05	1.2
40	2-OHE_1-6-N3Ade	419.1	Positive	420.0	135.9	26	10	1.41	2.4

[1] List of the 40 estrogen-related compounds with the masses of parent and daughter ions and the ionization mode that were used for MRM method optimization. The last column indicates the limit of detection for each compound.

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TABLE II

REPRESENTATIVE METABOLIC PROFILE OF A URINE SAMPLE OBTAINED FROM A HIGH RISK WOMAN.[1]

No.	Compound	pmole/mg creatinine mean, *n* = 2	Total pmole/mg creatinine
1	Androstenedione	1.56	1.56
2	Testosterone	0.24	0.24
3	E_{14} Sulfate	1.81	1.81
4	E_{24}	5.29	15.93
5	E_1	10.64	
6	2-OHE_2	3.09	3.15
7	2-OHE_1	0.05	
8	4-OHE_2	2.64	2.91
9	4-OHE_1	0.27	
10	16α-OHE_2	12.12	38.64
11	16α-OHE_1	26.52	
12	2-OCH_3E_2	1.95	49.81
13	2-OCH_3E_1	47.87	
14	4-OCH_3E_2	0.41	5.08
15	4-OCH_3E_1	4.67	
16	2-OH-3-OCH_3E_2	1.91	10.27
17	2-OH-3-OCH_3E_1	8.36	
18	2-OHE_2-1-SG	0.17	3.10^5
19	2-OHE_2-4-SG	0.17	
20	2-OHE_1-1-SG	0.49	
21	2-OHE_1-4-SG	0.47	
22	2-OHE_2-1+4-Cys	0.27	
23	2-OHE_1-1-Cys	0.10	
24	2-OHE_1-4-Cys	0.44	
25	2-OHE_2-1-NAcCys	0.07	
26	2-OHE_2-4-NAcCys	0.07	
27	2-OHE_1-1-NAcCys	0.43	
28	2-OHE_1-4-NAcCys	0.43	
29	4-OHE_2-2-SG	0.51	1.77^6
30	4-OHE_1-2-SG	0.50	
31	4-OHE_2-2-Cys	0.13	
32	4-OHE_1-2-Cys	0.06	
33	4-OHE_2-2-NAcCys	0.29	
34	4-OHE_1-2-NAcCys	0.28	
35	4-OHE_2-1-N7Gua	0.48	2.81

No.	Compound	pmole/mg creatinine mean, *n* = 2	Total pmole/mg creatinine
36	4-OHE_1-1-N7Gua	2.33	
37	4-OHE_2-1-N3Ade	137.78	137.90
38	4-OHE_1-1-N3Ade	0.13	
39	2-OHE_2-6-N3Ade	0.06	0.07
40	2-OHE_1-6-N3Ade	0.02	
(Ratio-4)[2] × 1,000		935	
(Ratio-2)[3] × 1,000		1	
(Ratio-4) + (Ratio-2) × 1,000			936

[1] Typically, each 2-ml urine sample was analyzed at least 2 times. The data obtained from LC/MS-MS were processed and normalized to creatinine levels. Since the E_1 and E_2 derivatives are interconvertible, the total amount for each E_1 plus E_2 derivative in the various categories are presented in the last column and used for calculating the final ratios of depurinating adducts to the respective metabolites and conjugates.

[2] $$\frac{4-\mathrm{OHE_1(E_2)}-1-\mathrm{N3Ade}+4-\mathrm{OHE_1(E_2)}-1-\mathrm{N7Gua}}{4-\text{catechol estrogens}+4-\text{catechol estrogen conjugates}} = \frac{\text{No. } 37+38+35+36}{\text{No. } 8+9+14+15+29 \text{ through } 34}$$

[3] $$\frac{2-\mathrm{OHE1\ (E2)}-6-\mathrm{N3Ade}}{2-\text{catechol estrogens}+2-\text{catechol estrogen conjugates}} = \frac{\text{No. } 39+40}{\text{No. } 6+7+12+13+16 \text{ through } 28}$$

[4] Free E_2 and E_1 in the urine sample.

[5] All 2-$OHE_1(E_2)$ conjugates.

[6] All 4-$OHE_1(E_2)$ conjugates.

TABLE III

URINARY LEVELS OF ESTROGEN COMPOUNDS IN HEALTHY WOMEN, HIGH-RISK WOMEN AND WOMEN WITH BREAST CANCER

No.	Compound	Control (n = 46)		High Risk (n = 12)			Breast Cancer (n = 18)		
		Median	Min–Max	Median	Min–Max	p-value[3]	Median	Min–Max	p-value[4]
1	Androstenedione	9.9	2.1–108	4.2	1.3–11.5	0.003[3]	5.5	0.4–95.1	0.047
2	Testosterone	2.2	0.2–16.5	0.8	0.2–2.8	**0.008**[5]	1.1	0.5–3.7	0.050
3	E_1-Sulfate	5.0	0.1–382	2.4	0.1–10.6	**0.087**	1.1	0.1–121	0.032
4	E_2	31.7	9.1–3865	11.4	4.7–80.0	**0.007**	28.1	3.6–151	0.943
5	E_1								
6	2-OHE_2	10.4	1.7–564	7.3	1.6–26.5	0.035	5.6	0.0–38.8	**0.006**
7	2-OHE_1								
8	4-OHE_2	12.4	2.4–157	8.1	2.9–43.3	0.138	5.2	0.0–28.0	**0.008**
9	4-OHE_1								
10	16α-OHE_2	168	10.3–638	33.7	0.0–279	**0.001**	10.9	0.0–86.3	<**0.001**
11	16α-OHE_1								
12	2-OCH_3E_2	49.7	4.7–568	31.1	6.5–452	0.275	26.8	2.2–171	0.044
13	2-OCH_3E_1								
14	4-OCH_3E_2	73.1	12.6–3979	5.9	1.6–37.1	<**0.001**	21.5	4.5–53.2	<**0.001**
15	4-OCH_3E_1								
16	2-OH-3-OCH_3E_2								
17	2-OH-3-OCH_3E_1								
18	2-OHE_2-1-SG	11.2[1]	0.8–79.8[1]	4.6[1]	1.2–18.7[1]	**0.005**[1]	3.1[1]	1.1–16.9[1]	**0.001**[1]
19	2-OHE_2-4-SG								
20	2-OHE_1-1-SG								
21	2-OHE_1-4-SG								
22	2-OHE_2-1+4-Cys								
23	2-OHE_1-1-Cys								
24	2-OHE_1-4-Cys								
25	2-OHE_2-1-NAcCys								

No.	Compound	Control (n = 46)		High Risk (n = 12)			Breast Cancer (n = 18)		
		Median	Min–Max	Median	Min–Max	p-value[3]	Median	Min–Max	p-value[4]
26	2-OHE$_2$-4-NAcCys								
27	2-OHE$_1$-1-NAcCys								
28	2-OHE$_1$-4-NAcCys								
29	4-OHE$_2$-2-SG	2.7[2]	0.7–24.6[2]	1.4[2]	0.6–6.8[2]	0.032[2]	1.3[2]	0.4–8.9[2]	0.027[2]
30	4-OHE$_1$-2-SG								
31	4-OHE$_2$-2-Cys								
32	4-OHE$_1$-2-Cys								
33	4-OHE$_2$-2-NAcCys								
34	4-OHE$_1$-2-NAcCys								
35	4-OHE$_2$-1-N7Gua	0.7	0.0–4.8	1.2	0.2–2106	0.238	1.6	0.4–11.8	**0.007**
36	4-OHE$_1$-1-N7Gua								
37	4-OHE$_2$-1-N3Ade	0.7	0.0–18.8	1.8	0.5–138	**0.007**	1.2	0.1–288	0.085
38	4-OHE$_1$-1-N3Ade								
39	2-OHE$_2$-6-N3Ade	0.1	0.0–6.5	0.1	0.0–0.7	0.999	0.1	0.0–5.4	0.960
40	2-OHE$_1$-6-N3Ade								

[1] All 2-OHE$_1$(E$_2$) conjugates.

[2] All 4-OHE$_1$(E$_2$) conjugates.

[3] Bonferroni-adjusted p-value for comparing control *versus* high risk using Mann–Whitney test.

[4] Bonferroni-adjusted p-value for comparing control *versus* breast cancer by using Mann–Whitney test.

[5] Significant p-values are shown in bold.

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TABLE IV

SUBJECT CHARACTERISTICS

Characteristic	Health status		
	Healthy (*n* = 37)	High risk (*n* = 12)	Breast cancer (*n* = 7)
Age at recruitment in years, mean (SD)	49 (7.85)	52 (6.09)	57 (12.16)
Age at menarche in years, mean (SD)	12 (1.45)	12 (1.44)	13 (1.25)
Menopausal Status, *n* (%)			
Premenopausal	17 (46%)	6 (50%)	3 (43%)
Postmenopausal	20 (54%)	6 (50%)	4 (57%)
Parity			
0	6 (16%)	0 (0%)	0 (0%)
1	3 (8%)	0 (0%)	0 (0%)
2	13 (35%)	7 (58%)	4 (57%)
3	11 (30%)	3 (25%)	0 (0%)
≥4	4 (11%)	2 (17%)	3 (43%)

TABLE V

RESULTS OF UNIVARIATE MULTIVARIATE LINEAR REGRESSION OF RATIO

Covariate	Univariate regression		Multivariate regression	
	Regression coefficient	*p*-value	Regression coefficient	*p*-value
Health status	103.60	0.005	108.56	0.007
Postmenopausal	35.66	0.51	41.18	0.44
Parity	15.01	0.42	−7.29	0.71