

## Epidemiological Study of Urinary 6 $\beta$ -Hydroxycortisol to Cortisol Ratios and Breast Cancer Risk<sup>1</sup>

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### Abstract

The ratio of urinary 6 $\beta$ -hydroxycortisol:cortisol is a measure of the activity of cytochrome p450 3A4 (CYP3A4). CYP3A4 catalyzes the formation of the genotoxic estrogen, 16 $\alpha$ -hydroxyestrene. It is also involved in the activation of many other mammary carcinogens, such as the polycyclic aromatic hydrocarbons and heterocyclic amines. We evaluated the association between urinary cortisol ratios and breast cancer risk in a subgroup of women who participated in a population-based case-control study in Shanghai. Overnight urine samples from 246 case-control pairs were assayed for 6 $\beta$ -hydroxycortisol (6 $\beta$ -OHC) to cortisol. The urine samples from all of the breast cancer patients were collected before any chemotherapy or radiotherapy. In-person interviews were conducted to obtain comprehensive information on dietary habits, reproductive history, and other lifestyle factors. The median levels of 6 $\beta$ -OHC:cortisol ratios were 2.61 in cases and 2.16 in controls, a 20.8% difference ( $P < 0.001$ ). The case-control difference was larger in women over 45 years of age (31.3% difference;  $P < 0.001$ ) than younger women (6.0%;  $P = 0.45$ ). After adjusting for confounding variables, the risks of breast cancer were increased from 1.0 (reference) to 1.6 [95% confidence interval (CI), 0.9–3.1], 2.2 (95% CI, 1.1–4.2), and 3.7 (95% CI, 1.9–7.4;  $P$  for trend,  $<0.001$ ) with increasing levels of 6 $\beta$ -OHC:cortisol ratios. The positive association was more pronounced among older women ( $>45$  years) than among younger women ( $\leq 45$  years). The adjusted odds ratios associated with the highest cortisol ratio were 6.0 (95% CI, 2.2–16.1) among older women and 2.2 (95% CI, 0.8–6.1) among younger women. The association

of the 6 $\beta$ -OHC:cortisol ratio was stronger among older women who had a high body mass index, late age at menopause, and early age at menarche (factors related to high endogenous estrogen exposure) than those who did not have these factors. These findings are consistent with the role of CYP3A4 in estrogen and carcinogen metabolism and suggest that high CYP3A4 activity may be a risk factor for breast cancer risk.

### Introduction

The ratio of urinary 6 $\beta$ -OHC<sup>3</sup>:cortisol has long been used as an indicator of the activity of CYP3As (1–6). The CYP3As are the most abundant P450s in the human liver, accounting for as much as 60% of the total P450 protein (7). The CYP3A enzymes catalyze the metabolism of over 50% of the drugs used in humans (7, 8). CYP3A4 is the major enzyme in the CYP3A family and catalyzes the metabolism of a variety of exogenous and endogenous compounds (7–9), including estrogen, a hormone that plays a central role in the etiology of breast cancer (10, 11). In humans, estrogens undergo extensive hydroxylation at positions C2, C4, C6, C15, and C16. Among them, estrogen C2, C16, and C4 hydroxylations are both biologically and quantitatively important (11, 12). These metabolic pathways are catalyzed by various forms of P450 (11). Many laboratories have shown that the 2-hydroxyestrogens exhibit virtually no peripheral estrogenic activity (11, 12), whereas the other two major estrogen metabolites, 16 $\alpha$ -hydroxyestrogens and 4-hydroxyestrogens, retain estrogenic activity and can also bind to DNA to form adducts (11–15). These observations would suggest that the 4-hydroxy and 16 $\alpha$ -hydroxy metabolites may have a role in the initiation of breast cancer. The levels of 16 $\alpha$ -hydroxyestrogens are elevated in mouse strains with a high frequency of mammary tumors (11). Similarly, the 4-hydroxyestrogens, but not the 2-hydroxyestrogens, have been found to induce tumors in animal models (11). Several recent studies (16–18) have shown that CYP3A4 plays a major role in the 4- and 16 $\alpha$ -hydroxylation of estrogens, particularly estrone, the predominant form of estrogens in postmenopausal women (10, 11). CYP3A4 is also involved in the activation of many environmental carcinogens, such as the polycyclic hydrocarbons, heterocyclic amines, aflatoxin, and nitrosamines (7–9, 19, 20). Some of these have been shown to be mammary carcinogens in experimental animals (21–25). Furthermore, CYP3A4 is present in human mammary epithelial cells (26), suggesting that this enzyme may be involved in the *in situ* activation of mammary carcinogens in these target cells.

Large interindividual variations in the activity of CYP3A4 have been reported (7, 8). Much of this variation is likely attributable to genetic polymorphisms in the gene that encodes

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<sup>3</sup> The abbreviations used are: 6 $\beta$ -OHC, 6 $\beta$ -hydroxycortisol; CYP3A, cytochrome P450–3A; OR, odds ratio; CI, confidence interval.

this enzyme and/or regulates the level of gene expression. A polymorphism in a regulatory element of the 5'-flanking region (-290 A→G) of the *CYP3A4* gene has been recently reported (27). This polymorphism was shown to be related to the risk of prostate cancer (27, 28) and treatment-related leukemia (29). Recently, we reported two additional allelic variants of the *CYP3A4* gene (30). However, all of the reported polymorphisms are rare in the Asian and Caucasian populations and unlikely to explain the interindividual enzyme variation observed in these populations (28–32). In a small, pilot case-control study conducted during 1996, we found a positive association between breast cancer risk and the ratio of urinary 6 $\beta$ -OHC:cortisol. To follow up this novel finding, we conducted a case-control study among a subgroup of women who have participated in the Shanghai Breast Cancer Study.

### Materials and Methods

The Shanghai Breast Cancer Study is a population-based, case-control study conducted among Chinese women in Shanghai (33, 34). With a population of over six million, it is the largest city on the east coast of China. This study was designed to recruit all of the incident breast cancer cases who were between the ages of 25 and 64 years between August of 1996 and March of 1998. A representative random sample of controls from the general population was also recruited. All of the cases and controls were permanent residents of urban Shanghai who had no prior history of cancer and were alive at the time of the interview. Through a rapid case-ascertainment system, supplemented by the population-based Shanghai Tumor Registry, 1601 eligible breast cancer cases were identified and in-person interviews were completed for 1459 subjects (91.1%). The major reasons for nonparticipation were refusal ( $n = 109$ ; 6.8%), death before interview ( $n = 17$ ; 1.1%), and inability to locate ( $n = 17$ ; 1.1%). The diagnosis of cancer was confirmed for all of the patients by two senior study pathologists through the review of tumor slides.

Controls were randomly selected from the female general population and frequency-matched to cases by age (5-year intervals). The number of controls in each age-specific stratum was determined in advance according to the age distribution of the incident breast cancer cases reported to the Shanghai Tumor Registry between 1990–1993. The Shanghai Resident Registry, which keeps registry cards for all of the adult residents in urban Shanghai, was used to randomly select controls. For each age-predetermined control, a registry card identifying a potential control from the same 5-year age group was randomly selected. Only women who lived at the listed address during the study period were eligible. In-person interviews were completed for 1556 (90.2%) of the 1724 eligible controls. Excluded from the study were 168 potential controls because of refusal ( $n = 166$ ; 9.6%) and death before the interview ( $n = 2$ ; 0.1%).

A structured questionnaire was used to elicit detailed information on demographic factors, menstrual and reproductive history, hormone use, dietary habits, prior disease history, physical activity, tobacco and alcohol use, weight, and family history of cancer. All of the interviews were tape-recorded for quality control purposes. All of the participants were weighed, and the circumferences of the waist and hip and their sitting and standing heights were determined. Of those who completed the in-person interviews, 2516 (83.1% of cases and 83.8% of controls) donated a blood sample, and 2995 (99.2% of cases and 99.5% of controls) donated a urine sample. All of the specimens were collected in the morning before any meals. To minimize the potential influence of breast cancer and its sequelae on the

levels of biomarkers in the blood and urine samples, specimens from breast cancer cases were collected as soon as possible after the initial diagnosis of cancer. Blood and urine samples were collected from about 50% of cases before any cancer therapy. Ascorbic acid (120 mg) was added to the urine (100 ml) to prevent oxidation of labile compounds. The samples were immediately placed in portable insulated cases with ice pads (0–4°C) and transported to the central laboratory for processing. All of the samples were aliquoted and stored at -70°C within 6 h after collection.

Although the Shanghai Breast Cancer Study used the frequency-matched design, for a subset of cases ( $n = 300$ ) whose blood and urine samples were collected before any chemotherapy or radiotherapy, we were able to individually match each case to a control on age ( $\pm 3$  years), menopausal status, and date of sample collection ( $\pm 30$  days). For the study of the association between urinary cortisol ratios and breast cancer, we included 250 pairs of cases and controls from this substudy using the individually matched design to reduce the costs. This approach also enhanced the comparability of cases and controls and eliminated between-assay variability by assaying samples from each case-control pair in the same batch.

The ratio of urinary 6 $\beta$ -OHC:cortisol was used as the indicator of *CYP3A4* activity (1–6). Urinary cortisol levels were determined by a cortisol RIA kit following the manufacturer's protocol (DiaSorin, Stillwater, MN). Briefly, 200  $\mu$ l of urine were extracted with 1 ml of methylene chloride. After phase separation, a 100- $\mu$ l aliquot from the bottom (methylene chloride) layer was added to tubes in which anti-cortisol rabbit serum was immobilized onto the inner wall. Standards or unknown samples were incubated with 1 ml (0.04 mCi) of cortisol tracer at 37°C for 45 min. After incubation, the contents of the tubes were aspirated, and the tubes were counted in a gamma counter (Packard Cobra). A standard curve was prepared for each run, and all of the assays were performed in duplicate.

The urinary 6 $\beta$ -OHC was determined by an enzyme immunoassay (Stabiligen, Nancy, France). 6 $\beta$ -OHC standards or urine samples (10  $\mu$ l) were added to 96-well microtiter plates containing specific 6 $\beta$ -OHC antibodies bound to the plate. Next, horseradish peroxidase conjugated to 6 $\beta$ -OHC was added, and the plates were incubated for 2 h at 25°C in the dark. After discarding the well contents and washing the plates four times with 0.05% Tween 20, the peroxide substrate was added. The color was allowed to develop in the dark for 45 min at 25°C. The reaction was terminated by the addition of H<sub>2</sub>SO<sub>4</sub>. The absorbance was read at 492 nm in a Titertek Multiskan plate reader. A standard curve was prepared for each run. All of the assays were performed in triplicate. The coefficients of variation for within-assay variability were 6.26% for cortisol and 5.86% for 6 $\beta$ -OHC. To eliminate the effect of between-assay variability (5.13% for cortisol and 18.62% for 6 $\beta$ -OHC) on the study results, the samples for each case-control pair were assayed in the same batch.

Because the data on the urinary 6 $\beta$ -OHC:cortisol ratios were skewed to high values, we used the Student *t* test for paired data to compare the mean differences between the cases and controls after a log-transformation of the data (35). The Wilcoxon signed rank test was used to compare the median difference between the cases and controls (35). To evaluate the strength of the association between the cortisol ratio and breast cancer risk, we performed a categorical analysis. The ORs and 95% CIs for women with intermediate or high cortisol ratios, compared to women with the lowest ratios, were determined using conditional logistic regression (36). Multivariate analyses were performed to adjust for potential confounding variables.

Table 1 Comparison of cases and controls by education levels and major breast cancer risk factors, Shanghai Breast Cancer Study, 1996–98

	No. of Cases (%) <sup>a</sup>	No. of Controls (%) <sup>a</sup>	OR (95% CI)
Education levels			
<Middle school	13.0	15.9	1.0 (reference)
Middle and high school	70.7	72.0	1.3 (0.7–2.4)
>High school	16.3	12.2	1.7 (0.8–3.5)
First-degree relative with breast cancer			
No	96.3	98.0	1.0 (reference)
Yes	3.7	2.0	1.8 (0.6–5.4)
Ever diagnosed with breast fibroadenoma			
No	93.5	95.9	1.0 (reference)
Yes	6.5	4.1	1.6 (0.7–3.5)
Waist-to-hip ratio			
<0.75	10.6	14.6	1.0 (reference)
0.75–0.84	66.7	69.1	1.4 (0.8–2.4)
≥0.85	22.8	16.3	2.2 (1.1–4.4)
Body mass index			
<20	10.6	16.3	1.0 (reference)
20–24	58.9	54.5	1.7 (1.0–2.9)
≥25	30.5	29.3	1.6 (0.9–3.0)
Age at menarche (yr)			
<13	9.8	10.2	1.0 (reference)
13–16	80.5	74.0	1.1 (0.6–2.0)
≥17	9.8	15.9	0.6 (0.3–1.4)
Age at menopause (yr) <sup>b</sup>			
<45	9.9	16.8	1.0 (reference)
45–54	81.2	76.8	2.4 (0.9–6.3)
≥55	8.9	6.3	3.2 (0.8–12.7)
Age at first live birth (yr) <sup>c</sup>			
<25	27.7	29.1	1.0 (reference)
25–34	66.5	68.8	1.0 (0.7–1.6)
≥35	5.8	2.1	2.9 (1.0–8.3)

<sup>a</sup> Unless otherwise specified, data are from 246 case-control pairs.

<sup>b</sup> Among postmenopausal women.

<sup>c</sup> Among parous women.

Trend tests were performed using logistic regressions after assigning the score  $j$  to the  $j$ -th level of the selected variable. All of the statistical analyses were based on two-tailed probabilities.

## Results

Of the 250 pairs of cases and controls assayed for CYP3A4 activity, complete data on cortisol ratios for 246 case-control pairs were available. The mean age was 48.4 years for cases and 48.2 years for controls. The educational level, however, was higher in the cases than the controls with 16.3% of the cases and 12.2% of the controls having received a college or higher educational level. Positive associations were observed for all of the major breast cancer risk factors, including family history of breast cancer, a prior history of breast fibroadenoma, high waist-to-hip ratio, high body mass index, early age of menarche, late age of menopause, and late age of first live birth (Table 1). The ORs, however, were statistically significant only for the high waist-to-hip ratio and older age at first live birth. In the multivariate analyses, we included for adjustments of potentially confounding effects of the following variables: education levels (<middle school, middle and high school, >high school); waist-to-hip ratio (<0.85, ≥0.85); body mass index (<20, ≥20); age at menarche (<16, ≥16); age at menopause (<45, ≥45); and age at first live birth (<35, ≥35).

The mean ratio of 6 $\beta$ -OHC:cortisol was 3.03 and 2.54 for

the cases and the controls, respectively (19.3% difference;  $P < 0.001$ ; Table 2). The case-control difference was more striking among women over 45 years of age (28.1% difference;  $P < 0.001$ ) than among younger women (8.5% difference;  $P = 0.33$ ). Similar patterns were observed when medians for cases and controls were compared (Table 2).

To estimate the risk of breast cancer associated with high cortisol ratios, cases and controls were categorized into four groups according to the quartile distribution of the cortisol ratio among controls (Table 3). After adjusting for all of the potential confounding variables, a dose-response relationship was observed between the cortisol ratio and breast cancer risk ( $P$  for trend; <0.001), with a nearly 4-fold elevated risk (95% CI, 1.9–7.4) observed for women in the highest cortisol ratio group. The association was stronger among older women (OR, 6.0; 95% CI, 2.2–16.1) than among younger women (OR, 2.2; 95% CI, 0.8–6.1), and the dose-response relationship was statistically significant only among older women ( $P$  for trend, <0.001). Similar analyses were performed among 173 breast cancer cases diagnosed at an early stage (0–II) and their individually matched controls. The same pattern of association was observed in this subset of cases and controls as that presented in Table 3. The dose-response relationship was observed only among older women ( $P$  for trend, 0.003), with adjusted ORs of 1.0 (reference), 1.7 (95% CI, 0.6–4.9), 3.1 (95% CI, 0.9–10.6), and 6.9 (95% CI, 1.8–26.2) with increasing quartile of cortisol ratios.

Additional analyses were performed among older women to evaluate potential joint effects of urinary cortisol ratios and factors related to endogenous estrogen exposure (Table 4). The tertile instead of quartile distribution of the cortisol ratio was used to group cases and controls to enhance the stability of risk estimates. A dose-response relationship was observed in all of the strata defined by body mass index, age at menarche, and age at menopause. The association was stronger among those who had a high body mass index, younger age at menarche, and older age at menopause than those who did not have these risk factors. A test for multiplicative interaction was statistically significant for the joint effect of the cortisol ratio and body mass index ( $P = 0.046$ ).

## Discussion

We have found in this case-control study that there is a strong association between the risk of breast cancer and urinary 6 $\beta$ -OHC:cortisol ratios, a measure of CYP3A4 activity. Furthermore, the risk increased in a dose-response manner with increasing cortisol ratios, particularly among older women. These findings are novel and consistent with the role of CYP3A4 in estrogen metabolism and carcinogen activation.

The urinary 6 $\beta$ -OHC:cortisol ratio has long been used as a noninvasive indicator of hepatic P450 activity (1–6). The level of 6 $\beta$ -OHC is closely correlated with the expression of human hepatic CYP3A4 (6) and is significantly increased by typical CYP3A4 inducers, such as rifampin (6). However, there is a poor correlation between this index and other indices of hepatic CYP3A4 activity, such as the erythromycin breath test and the dapsone recovery ratio (37). On the other hand, these two tests also do not correlate with each other. Furthermore, both of them require the administration of the drug probes and repeated sample collections. Hence, it is much more difficult to implement these probes in population-based epidemiological studies than the cortisol ratio test (37).

The absence of a good correlation among the three indices for CYP3A4 activity may reflect differences in the organs that

Table 2 Levels of urinary 6 $\beta$ -OHC:cortisol ratios for cases and controls, Shanghai Breast Cancer Study, 1996–98

	Urinary cortisol ratios		Percentage difference <sup>a</sup>	<i>P</i> <sup>b</sup>
	Case	Control		
All of the subjects (246 pairs) <sup>c</sup>				
Mean $\pm$ SD	3.03 $\pm$ 1.88	2.54 $\pm$ 1.68	19.3	0.001
Median (25 <sup>th</sup> , 75 <sup>th</sup> percentile)	2.61 (1.63, 3.92)	2.16 (1.32, 3.28)	20.8	<0.001
Age $\leq$ 45 years (102 pairs) <sup>c</sup>				
Mean $\pm$ SD	2.95 $\pm$ 1.89	2.72 $\pm$ 1.66	8.5	0.33
Median (25 <sup>th</sup> , 75 <sup>th</sup> percentile)	2.49 (1.53, 3.85)	2.35 (1.48, 3.59)	6.0	0.45
Age >45 years (144 pairs) <sup>c</sup>				
Mean $\pm$ SD	3.10 $\pm$ 1.88	2.42 $\pm$ 1.68	28.1	<0.001
Median (25 <sup>th</sup> , 75 <sup>th</sup> percentile)	2.64 (1.74, 3.99)	2.01 (1.24, 2.95)	31.3	<0.001

<sup>a</sup> Expressed as (mean<sub>cases</sub> – mean<sub>controls</sub>)/mean<sub>controls</sub> or (median<sub>cases</sub> – median<sub>controls</sub>)/median<sub>controls</sub>.

<sup>b</sup> *P*s were from paired *t*-test of log-transformed data (for mean comparisons) or the Wilcoxon signed rank test (for median comparisons).

<sup>c</sup> Case-control pairs.

Table 3 Association of breast cancer risk with urinary ratios of 6 $\beta$ -OHC:cortisol, Shanghai Breast Cancer Study, 1996–98

Cortisol ratios (by quartiles)	No. of Cases	No. of Controls	Adjusted OR <sup>a</sup>	95% CI
All Women				
$\leq$ 1.32	39	62	1.0	
1.33–2.16	51	60	1.6	0.9–3.1
2.17–3.28	63	62	2.2	1.1–4.2
$\geq$ 3.28	93	62	3.7	1.9–7.4
Trend test			<i>P</i> < 0.001	
Women $\leq$ 45 years				
$\leq$ 1.32	16	21	1.0	
1.33–2.16	26	24	1.5	0.6–3.9
2.17–3.28	21	26	1.1	0.4–3.0
$\geq$ 3.28	39	31	2.2	0.8–6.1
Trend test			<i>P</i> = 0.25	
Women >45 years				
$\leq$ 1.32	23	41	1.0	
1.33–2.16	25	36	1.4	0.6–3.5
2.17–3.28	42	36	3.1	1.2–7.7
$\geq$ 3.28	54	31	6.0	2.2–16.1
Trend test			<i>P</i> < 0.001	

<sup>a</sup> Adjusted for education level, waist-to-hip ratio, body mass index, age at menarche, age at menopause, and age at first live birth.

metabolize erythromycin, dapson, and cortisol (37). Erythromycin *N*-demethylation is primarily limited to the liver, whereas a portion of the 6 $\beta$ -OHC may be produced in extrahepatic organs, such as the kidney (37, 38). In particular, CYP3A5, the predominant CYP3A isoform found in the renal collecting tubules (38), has been shown to specifically catalyze cortisol 6 $\beta$ -hydroxylation (39). As a result, the urinary 6 $\beta$ -OHC:cortisol ratios may be a measure of the collective activities of several related enzymes. Because cortisols, like estrogens, are endogenous steroid hormones that are chemically more closely related to estrogens than erythromycin and dapson, the ratio of 6 $\beta$ -OHC:cortisol may be a better measure than the other two indices of the combined CYP3A4 and CYP3A5 activities that catalyze estrogen hydroxylation.

Urinary excretion of 6 $\beta$ -OHC and cortisol fluctuates during the course of the day, a reflection of the circadian rhythm of cortisol secretion. However, the urinary ratios of the two steroids remain constant (2). In a recent study of 26 women over 28 days, the mean weekly 6 $\beta$ -OHC:cortisol ratio was found to be very stable over time (40). Furthermore, the investigators observed no significant differences in the ratios between pre- and postmenopausal women or among premeno-

pausal women over the menstrual cycle (40). In our study, we also collected a second urine sample from 17 controls about 2 months after their initial urine collection. The cortisol ratios measured from the first and second urine samples were highly correlated ( $r = 0.79$ ;  $P < 0.001$ ), indicating that a single measurement of the cortisol ratio reflects the long-term levels of this ratio.

Although no previous studies have reported an association between CYP3A4 activity and breast cancer risk, CYP3A4 phenotypes have been investigated in relationship to cancer of the liver and bladder (41, 42). In a hospital-based, case-control study of liver cancer, Ng *et al.* (41) found that patients with nonresectable hepatocellular carcinoma had a significantly higher level of 6 $\beta$ -OHC:cortisol ratios than controls. On the other hand, Fleming *et al.* (42) showed that, based on the dapson recovery ratios, bladder cancer patients had lower CYP3A4 activities than controls. These findings are biologically plausible, because CYP3A4 has been reported to be involved in the metabolism of aflatoxin, arylamines, and nitrosamines (7–9, 19, 20, 43), the presumed carcinogens involved in the initiation of liver and bladder tumors. Finally, Lin *et al.* (44) recently reported that the mean urinary 6 $\beta$ -OHC:cortisol ratios were substantially higher in Caucasian women than in their Asian counterparts. This is consistent with the observation of a higher incidence of breast cancer in the former than in the latter population. The mean ratios reported in that study for Asian women were 2.2 to 2.8 (44), which are very close to the mean of 2.4 observed in our study.

The positive association between urinary cortisol ratios and breast cancer risk may be attributable to the role of CYP3A4 in the 16 $\alpha$ -hydroxylation of estrone or activation of mammary carcinogens. Several lines of evidence implicate 16 $\alpha$ -hydroxyestrone in breast carcinogenesis (11–15, 45–48). First, 16 $\alpha$ -hydroxyestrone may induce genotoxic DNA damage through covalent binding to DNA (11, 14, 15, 45). Secondly, this genotoxic metabolite is elevated in explant cultures of mammary terminal duct lobular units from breast cancer patients (46). Finally, although not yet consistent (47–50), several case-control studies (47–49), including our own study in Shanghai (49), have shown that 16 $\alpha$ -hydroxyestrone levels are higher in breast cancer patients than in controls. Recent studies (16–18) have suggested that CYP3A4 may have a major role in the 16 $\alpha$ -hydroxylation of estrone. In particular, Shou *et al.* (16) reported that CYP3A4 had at least a 5-fold greater activity in estrone 16 $\alpha$ -hydroxylation than CYP1A2, 2B6, 2C6, 2C9, 2C9R114C, and 2E1. Furthermore, a recent study (18) reported that the hepatic CYP3A4/5 activity was closely correlated with

Table 4 Interaction of the urinary ratio of 6 $\beta$ -OHC:cortisol with body mass index and menstrual factors on the risk of breast cancer, Shanghai Breast Cancer Study, 1996–98

	OR <sup>a</sup> (95% CI) by cortisol ratios (by tertile)		
	≤1.52	1.53–2.77	>2.77
Body mass index (by median) <sup>b</sup>			
≤23.95	1.0 (reference)	3.5 (1.1–11.0)	3.8 (1.2–12.0)
>23.95	1.1 (0.4–3.3)	1.5 (0.5–4.4)	8.3 (2.4–28.4)
		<i>P</i> for interaction, 0.046	
Age at menarche <sup>b</sup>			
≥15 years	1.0 (reference)	1.9 (0.7–4.8)	3.1 (1.1–8.6)
<15 years	1.0 (0.3–3.2)	1.6 (0.6–4.6)	6.6 (2.2–20.3)
		<i>P</i> for interaction, 0.32	
Age at menopause <sup>c</sup>			
<50	1.0 (reference)	1.6 (0.4–6.2)	3.7 (0.9–14.4)
≥50	1.5 (0.4–5.2)	2.4 (0.6–9.3)	6.0 (1.4–25.5)
		<i>P</i> for interaction, 0.99	

<sup>a</sup> Adjusted for education level, waist-to-hip ratio, age at first live birth, and two of the following three variables that were not evaluated as the stratified variable: body mass index, age at menarche, and age at menopause.

<sup>b</sup> Analyses were restricted to 144 pairs of cases and controls who were over 45 years of age.

<sup>c</sup> Based on 92 pairs of cases and controls who had natural menopause and were over 45 years of age.

the formation of 16 $\alpha$ -hydroxyestrone. Our findings for a positive association between urinary cortisol ratio and breast cancer risk, particularly among those who had risk factors related to endogenous estrogen exposure, are consistent with the hypothesis that elevated synthesis of 16 $\alpha$ -hydroxylation estrone may increase the risk of breast cancer.

The stronger association between the cortisol ratio and breast cancer among older rather than younger women might be expected because it is likely that high CYP3A4 activities lead to the development of breast carcinogenesis through a cumulative effect over decades of estrogen exposure on breast tissue. Alternatively, the difference in the strength of the association between pre- and postmenopausal women could be attributable to differences in the predominant estrogens in the two groups. In particular, estrone is relatively unimportant in premenopausal women, although it is the predominant estrogen in postmenopausal women (10, 11). Because CYP3A4 catalyzes the 4- and 16 $\alpha$ -hydroxylation of estrone, it would be expected that higher activities of this enzyme would have a greater effect in postmenopausal women than in younger women (16–18). Finally, in general, only the mutation of high-penetrance genes, such as *BRCA1/2*, *P53*, and *ATM*, are thought to be important in the etiology of breast cancer among younger women (51).

A potential problem with this study is that the urine samples used in the assay were collected after the diagnosis of the cancer. This could have affected the levels of both the urinary cortisol and its metabolite, and thus their ratios could be relatively unchanged. To minimize the potential influence of breast cancer and its sequelae on the levels of urinary analytes, we attempted to collect urine samples as soon as possible after their initial cancer diagnosis. As a result, all of the urine samples from the cases included in this report were collected before any chemotherapy or radiotherapy and within days after initial breast cancer diagnosis. We also analyzed data collected from early stage breast cancer cases and their controls and found a similar positive association between the cortisol ratio and breast cancer risk as observed in all of the subjects combined. Therefore, it is unlikely that the positive association observed in this study can be explained by the effect of breast cancer and related treatment.

In summary, in this study we have shown for the first time that urinary 6 $\beta$ -OHC:cortisol ratio is a potential biomarker for breast cancer risk, particularly in older women. These findings

are biologically plausible and consistent with the role of CYP3A4 in estrogen and carcinogen metabolism.

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