Urinary Excretion of Phytoestrogens and Risk of Breast Cancer among Chinese Women in Shanghai[1]

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Abstract

Although the majority of ecological and experimental studies have suggested a potential role of phytoestrogens in breast cancer prevention, findings from epidemiological studies have been inconsistent. Part of the inconsistencies may be attributable to the difficulty in measuring intake levels of phytoestrogens. Overnight urine samples from 250 incident breast cancer cases and their individually matched controls were analyzed for urinary excretion rates of isoflavonoids, mammalian lignans, and citrus flavonoids. The study subjects were a subset of the participants in the Shanghai Breast Cancer Study, a large population-based case-control study conducted in Shanghai from 1996–1998. To minimize potential influence of treatment on the exposure of interest, urine samples from breast cancer cases were collected before cancer therapy. Urinary excretion of total isoflavonoids and mammalian lignans was substantially lower in breast cancer cases than in controls. The median excretion rate of total isoflavonoids was 13.97 nmol/mg creatinine in cases and 23.09 in controls ($P = 0.01$), and the median excretion rate of total lignans was 1.77 in cases and 4.16 in controls ($P < 0.01$). The risk of breast cancer was reduced with increasing excretion of total isoflavonoids (*P* for trend, 0.04) and total lignans (*P* for trend, <0.01), with adjusted odds ratios of 0.62 (95% confidence interval, 0.39–0.99) and 0.40 (95% confidence interval, 0.24–0.64) observed for the highest *versus* the lowest tertile of total isoflavonoid and lignan excretion, respectively. The adjusted odds ratio was 0.28 (95% confidence interval, 0.15–0.50) for women who had a high excretion rate of both total lignans and isoflavonoids compared with those with a low excretion of both groups of phytoestrogens. No association was observed with citrus flavonoids. The results from this study suggest that high intake of certain phytoestrogens may reduce the risk of breast cancer.

Introduction

Evidence from ecological and experimental studies has suggested that high consumption of phytoestrogens, especially soyfood isoflavones, may reduce the risk of breast cancer (1–3). The majority of previous epidemiological studies on phytoestrogens have focused on the evaluation of the association between usual intake of soyfoods and breast cancer risk, and the results have been inconsistent (4, 5). A major concern for previous studies may be the validity of the food questionnaire in assessing usual soyfood intake, because none of the previous studies was designed specifically to investigate the association between soyfood intake and breast cancer risk (4).

Phytoestrogens are plant-derived, organic, nonsteroidal molecules possessing a weak estrogenic or antiestrogenic activity. They have been shown to have inhibitory effects on hormone-related cancers in many *in vitro* and *in vivo* systems (1). The principal groups of dietary phytoestrogens consist of: (*a*) isoflavones, to which humans are exposed almost exclusively through soyfood intake; (*b*) lignans, which humans ingest mostly from whole grains, seeds, and some fruits and vegetables (1); and (*c*) flavonoids, found ubiquitously in vascular plants (6). After consumption, phytoestrogens undergo many metabolic conversions by intestinal bacteria, and both the metabolites and parent compounds can be absorbed into the blood and then excreted, mainly in urine (1). A substantial interindividual variation in bioavailable phytoestrogens has been observed after ingestion of isoflavones and lignans (7, 8). Therefore, urinary excretion of phytoestrogens may be a more accurate measure of bioavailability of these phytoestrogens than dietary assessment. Urinary excretion of phytoestrogens, however, reflects primarily intake levels of soyfoods over the past 24–96 h (7). In our previous study, we found a clear dose-response relationship between usual soy protein intake and urinary excretion rate of isoflavonoids in a spot urine (9), strongly suggesting that urinary isoflavonoids can be used as a measure of usual soy intake among Chinese women in Shanghai, a population with a persistently high consumption of soyfoods. An inverse association between urinary excretion of some phytoestrogens and the risk of breast cancer has been implicated by four studies (10–13), including our early study in Shanghai (11). The results from previous studies, however, have been inconsistent, and none of these studies has examined the associations by menopausal status because of a small sample size (10–13). A recent cohort study included only one isoflavonoid and lignan, and the study was conducted among a

Received 8/24/01; revised 5/3/02; accepted 5/17/02.
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[1] This work was supported by USPHS Grants CA64277 and CA86119 and supplemental CCSG support CA71789-03. Portions of this work constituted partial fulfillment for a Ph.D. in Epidemiology at the University of South Carolina by Q. D.
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population with very low intake of isoflavones (13). Of all previous studies reported thus far, our earlier study was the only one that investigated all major isoflavonoids (11). This early study, however, included only 60 case-control pairs and did not report the results on lignans and other phytoestrogens. No study has evaluated the association between citrus flavonoids, another group of phytoestrogens (14, 15), and breast cancer risk. Recently, we have improved our laboratory methods in phytoestrogen detection and examined comprehensively the associations between breast cancer risk and urinary excretion of isoflavonoids, lignans, and citrus flavonoids in a larger study, using data and specimens collected in the Shanghai Breast Cancer Study.

Materials and Methods

Subject Selection. The Shanghai Breast Cancer Study is a population-based case-control study conducted among Chinese women in urban Shanghai during 1996–1998. The detailed study methods have been reported elsewhere (11, 16). In brief, the study was designed to recruit all women, 25–64 years of age, who were newly diagnosed with breast cancer between August 1996 and March 1998 and a group of controls randomly selected from the general population. All study subjects were permanent residents of urban Shanghai with no prior history of cancer. The use of human subjects in this study was approved by relevant institutional review boards. Through a rapid case-ascertainment system, supplemented by the population-based Shanghai Tumor Registry, 1602 eligible breast cancer cases were identified during the study period, and in-person interviews were completed for 1459 patients (91.1%). The major reasons for nonparticipation were refusal (109 cases, 6.8%), death prior to interview (17 cases, 1.1%), and inability to locate (17 cases, 1.1%). Two senior pathologists reviewed all slides to confirm all cancer diagnoses.

The Shanghai Resident Registry, which keeps all records for permanent residents in urban Shanghai, was used to select controls randomly from female residents, frequency-matched to cases by age (5-year interval). 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 from 1990 to 1993. Only women who lived at the address identified during the study period were considered to be eligible for the study. In-person interviews were completed for 1556 (90.3%) of the 1724 eligible controls identified. Reasons for nonparticipation included refusal (166 controls, 9.6%) and death or a prior cancer diagnosis (2 controls, 0.1%).

Data and Specimen Collection. All study participants were interviewed in person by trained interviewers and measured for weight, circumferences of waist and hip, and sitting and standing heights. 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. Information on usual dietary intake was collected using a comprehensive quantitative food frequency questionnaire, including questions that ascertain usual consumption of soymilk, tofu, dry soybeans, soy products other than tofu, fresh soybeans, and soybean sprouts (17). These six food items account for >90% of soyfoods consumed in Shanghai.

Among those who completed the interviews and anthropometrics, 83.1% of cases and 83.8% of controls donated a blood sample, and 99.2% of cases and 99.5% of controls donated a urine sample. All specimens were collected in the morning before breakfast. To minimize potential influence of breast cancer and its sequelae on the levels of biomarkers in blood and urine samples, specimens from breast cancer cases were collected as soon as possible after initial cancer diagnosis. For nearly 50% of the cases, biospecimen collections and in-person interviews were completed before any cancer therapy. Ascorbic acid (125 mg/100-ml urine sample) was added to prevent degradation of labile compounds. Immediately after collection, samples were placed in a portable insulated case with ice pads (0–4°C) and transported to the central laboratory for processing. All samples were aliquoted and stored at −70°C within 6 h after collection.

To increase the comparability between cases and controls for molecular epidemiological studies, an individually matched case-control study (500 pairs) was built into the Shanghai Breast Cancer Study. This substudy included cases whose blood and urine samples were collected before any cancer treatment and their controls, who were individually matched to the index cases by age (± 3 years), menopausal status (yes, no), and date of sample collection (±30 days). For the current study of urinary phytoestrogens, 250 case-control pairs were selected, and their urine samples were assayed for isoflavonoids, lignans, and citrus flavonoids. To eliminate the effect of between-assay variability on study results, samples for each case-control pair were assayed in the same batch.

Lab Assays of Urinary Phytoestrogens and Creatinine. Extraction of urines was performed as reported previously (7), but analysis was carried out by LC/MS.[3] In brief, urine was thawed, stirred, and centrifuged, followed by mixing 0.25 ml of clear supernatant with 50 μl of 0.5 M triethylamine acetate (pH 7.0), 5 μl of β-glucuronidase, and 5 μl of arylsulfatase and incubating for 1 h at 37°C. This mixture was extracted three times with 2.0 ml of ethyl ether. After centrifugation, the organic phases were combined, followed by drying under nitrogen. The dry extract was redissolved in 0.25 ml of methanol by vortexing, followed by mixing with 0.25 ml of 0.2 M acetate buffer (pH 5). The resulting clear solution was then analyzed immediately by injecting 20 μl into the LC/MS system or stored at −20°C until analyzed. LC/photo diode array/MS was carried out with a Spectra-Physics, Inc.-designed quaternary solvent delivery liquid chromatography system with multiple channel diode-array detection and a quadrupole ion trap mass spectrometer model LCQ equipped with an electrospray ionization unit (Thermo Finnigan Corp., San Jose, CA). LC analysis was performed as developed recently by using a HydroBond PS 18 (100 × 3.0 mm; 5 μm) reversed phase column coupled to a HydroBond PS C_{18} (25 × 3.2 mm; 5 μm) direct-connect guard column with the following linear gradient at a flow rate of 0.25 ml/min: methanol:acetonitrile:0.5% aqueous acetic acid from 20:20:60 to 35:35:30 in 13 min, followed by holding at 35:35:30 for 1 min, changing to 45:45:10 in 1 min, followed by holding there for 4 min, and changing to 20:20:60 in 1 min with equilibration for 5 min before subsequent injection. Mass spectrometric measurements were performed in the negative mode after electrospray ionization with mass screening covering the range 180–350 amu. Capillary temperature was held at 260°C; sheath nitrogen gas flow and auxiliary nitrogen gas flow was set at 90 and 30 units corresponding to approximately 90 and 10 PSI, respectively. ESI source needle voltage was set at 4.6 kV,

[3] The abbreviations used are: LC/MS, liquid chromatography mass spectrometry; OR, odds ratio; CI, confidence interval; BMI, body mass index; WHR, waist:hip ratio; O-DMA, *O*-desmethylangolensin.

leading to an average current of 80 μA, and capillary voltage was set at −8 V. The divert valve was programmed to allow eluate flow into the mass spectrometer from 4.3 to 17.9 min of the high-performance liquid chromatography run time. Individual analytes were monitored by screening M − 1 masses (mass of molecular ion minus hydrogen) of analytes alone or in combination with selected reaction monitoring of first-generation daughters applying collision energies of 40–42% as follows: daidzein with 2 amu mass width at M − 1 = *m*/*z* 253; dihydrodaidzein with 2 amu mass width at parent mass of *m*/*z* 255; glycitein with 2 amu mass width at M − 1 = *m*/*z* 283 and first-generation daughter mass of *m*/*z* 268; genistein with 4 amu mass width at *m*/*z* 270 (parent mass, *m*/*z* 269) and first-generation daughter masses of *m*/*z* 201, 225, 241, and 269; dihydrogenistein with 4 amu mass width at *m*/*z* 270 (parent mass, *m*/*z* 271) and first-generation daughter mass of *m*/*z* 165; O-DMA with 2 amu mass width at M − 1 = *m*/*z* 257; formononetin (internal standard) with 2 amu width at M − 1 = 267 and first-generation daughter mass of *m*/*z* 252; hesperetin with 2 amu mass width at M − 1 = *m*/*z* 301 and first-generation daughter masses of *m*/*z* 283, 257, and 242; naringenin with 2 amu mass width at M − 1 = *m*/*z* 271 and first-generation daughter masses of *m*/*z* 177 and 151; enterodiol with 2 amu mass width at M − 1 = *m*/*z* 301 and first-generation daughter masses of *m*/*z* 283 and 253; enterolactone with 2 amu mass width at M − 1 = *m*/*z* 297 and first-generation daughter masses of *m*/*z* 253 and 189. Detection limits of this method for urinary levels of daidzein, dihydrodaidzein, glycitein, genistein, dihydrogenistein, O-DMA, hesperetin, naringenin, enterodiol, and enterolactone were 27, 39, 1.5, 4.2, 1.8, 27, 5.8, <1, 2.0, and 1.9 nM, respectively, on the basis of a signal:noise ratio of 3. Mean intraassay variability of this method from 41 repeats for detecting urinary phytoestrogens was 7.4% for daidzein (4714 nM), 11.4% for dihydrodaidzein (166 nM), 5.2% for glycitein (587 nM), 6.4% for genistein (3801 nM), 8.2% for dihydrogenistein (479 nM), 17.3% for O-DMA (13 nM), 14.8% for hesperetin (104 nM), 7.0% for naringenin (408 nM), 17.1% for enterodiol (48 nM), and 8.8% for enterolactone (366 nM), respectively. Interassay variability measured with six repeats of quality control samples was 7.2% for daidzein, 14.8% for dihydrodaidzein, 8.5% for glycitein, 11.8% for genistein, 18.9% for dihydrogenistein, 60.0% for O-DMA, 21.0% for hesperetin, 7.3% for naringenin, 8.2% for enterodiol, and 8.9% for enterolactone, respectively. In addition, when levels exceeded the MS calibration curve, analytes were monitored at 200–400 nm with photo-diode array detection and were quantitated with this method as described previously (7). Quantitation was performed using peak areas after adjustment for internal standard recovery. Creatinine levels were determined with a test kit (kit 555l; Sigma Co., St. Louis, MO), based on the Jaffe reaction (11), and mean coefficients of variation for intra- and interassay variability were found to be 4.1 and 6.7%, respectively.

Data Analyses. Phytoestrogens in urine were expressed in nanomol/mg creatinine by adjusting phytoestrogen concentrations for urinary creatinine levels. Because the data were skewed to high value, log-transformed data were used in the paired Student *t* tests to compare the mean differences between cases and controls. The Wilcoxon signed rank test also was used for comparisons of the median difference between cases and controls. To evaluate the potential dose-response relationship between urinary excretion rates of phytoestrogens and breast cancer risk, cases and controls were categorized into three groups according to the tertile distribution of urinary excretion rates of these compounds among controls. ORs and 95% CIs for the upper two tertile groups were derived using conditional logistic regression, compared with the lowest tertile group (18). Stratified analyses were used to assess potential effect modifiers. Multivariate analyses were performed to adjust for potential confounding variables. Tests for trend across tertiles were performed in logistic regressions by assigning the score *j* to the *j*th level of the variable selected. Further analyses were stratified by menopausal status. All statistical analyses were based on two-tailed probability.

Table 1 Comparison of cases and controls by selected demographic and risk factors, Shanghai, 1996–1998

Characteristic[a]	Cases (n = 250)	Controls (n = 250)	P^b
Demographic factors			
Age (yr)	49.1 ± 8.5	48.9 ± 8.7	0.85
Education (%)			
Elementary and under	16.0	18.0	
Middle and high school	70.4	68.4	
College and high	13.6	13.6	0.83
Non-dietary risk factors			
First-degree relative with breast cancer (%)	3.6	1.6	0.16
Ever diagnosed with fibroadenoma (%)	10.4	3.2	<0.01
BMI	23.6 ± 3.4	22.6 ± 3.5	0.08
Body weight	59.4 ± 9.0	57.7 ± 8.8	0.03
WHR	0.81 ± 0.05	0.80 ± 0.05	0.14
Exercise regularly (%)	22.8	33.2	0.01
Age at menarche	14.7 ± 1.7	14.9 ± 1.8	0.20
Age at menopause (yr)[c]	48.8 ± 4.2	48.2 ± 4.4	0.26
Nulliparity	5.2	4.0	0.52
Number of live birth[d]	1.6 ± 0.9	1.6 ± 0.9	0.87
Age at first live birth (yr)[d]	26.7 ± 4.3	26.1 ± 4.2	0.09
Nutritional factors			
Total energy intake (kcal/day)	1919.8 ± 476.6	1847.1 ± 419.7	0.07
Total fat intake (g/day)[e]	36.09 ± 0.77	37.31 ± 0.77	0.26
Total fruit and vegetable intake (g/day)[e]	481.6 ± 15.8	473.7 ± 15.8	0.72
Soy protein intake (g/day)[e]	10.69 ± 0.61	11.49 ± 0.61	0.36
Total meat (g/day)[e]	92.59 ± 3.44	83.42 ± 3.44	0.06

[a] Unless otherwise specified, mean ± SD are presented.
[b] *P*s were from the χ^2 test (for categorical variables) or Wilcoxon signed-rank test (for continuous variables).
[c] Among postmenopausal women.
[d] Among ever-pregnant women.
[e] Adjusted for total energy intake.

Results

Table 1 shows comparisons of cases and controls on demographic factors and known lifestyle risk factors of breast cancer, including dietary factors. Compared with controls, cases had an earlier age at menarche, older age at menopause, and older age at first live birth. Cases also were more likely to have higher education, a family history of breast cancer among first-degree relatives, a history of breast fibroadenoma, a higher BMI, weight and WHR, and to be nulliparous. They were less likely to exercise regularly. These results are consistent with the results obtained from all subjects in the Shanghai Breast Cancer Study (16) and studies in other populations (19, 20). Although not statistically significant, cases tended to have a higher energy intake than controls. After adjusting for total energy intake, cases had a slightly lower mean intake level of fat or soy protein, but higher mean intake of total meat or fruits and vegetables. The differences, however, were reduced after ad-

Table 2 Comparison of urinary excretion of isoflavonoids, lignans, and citrus flavonoids between breast cancer cases and controls, Shanghai, 1996–1998

Urinary excretion rate[a]	Mean ± SD		% of difference[b]	*P* from paired *t* test[c]	Median (25th, 75th percentile)		% of difference[b]	*P* from Wilcoxon test
	Cases	Controls			Cases	Controls		
Total isoflavonoids[d]	32.32 ± 43.70	40.50 ± 62.55	−20.1	<0.01	13.97 (3.54, 44.65)	23.09 (7.07, 53.50)	−39.5	0.01
Parent compounds	20.98 ± 28.84	27.75 ± 37.73	−24.4	<0.01	8.17 (2.41, 33.26)	15.71 (4.61, 35.99)	−48.0	<0.01
Daidzein	12.65 ± 18.14	17.27 ± 28.26	−26.7	<0.01	4.95 (1.45, 19.45)	9.90 (3.03, 21.61)	−50.0	<0.01
Genistein	6.65 ± 9.69	8.04 ± 9.88	−17.3	0.02	2.19 (0.44, 9.36)	4.37 (0.78, 11.43)	−49.9	0.07
Glycitein	1.68 ± 2.82	2.43 ± 3.64	−30.9	<0.01	0.46 (0.11, 2.10)	1.17 (0.28, 2.90)	−60.7	<0.01
Metabolites	11.33 ± 20.40	12.75 ± 29.73	−7.0	0.01	3.12 (0.83, 130)	5.05 (1.45, 12.48)	−38.2	0.13
O-DMA	3.88 ± 7.04	5.03 ± 20.38	−22.9	0.28	1.05 (0.25, 3.75)	1.41 (0.46, 4.05)	−25.5	0.40
Dihydrodaidzein	5.32 ± 11.76	5.23 ± 9.22	1.7	0.35	0.81 (0.00, 5.40)	1.67 (0.18, 5.68)	−51.5	0.17
Dihydrogenistein[a]	2.13 ± 8.11	2.49 ± 8.59	−14.5	0.09	0.13 (0.02, 0.84)	0.27 (0.05, 1.13)	−51.9	0.13
Lignans	4.44 ± 7.43	7.18 ± 9.25	−38.2	<0.01	1.77 (0.34, 5.22)	4.16 (0.82, 8.56)	−57.5	<0.01
Enterolactone	3.59 ± 5.64	6.28 ± 8.64	−42.8	<0.01	1.34 (0.23, 4.25)	3.40 (0.50, 7.56)	−60.6	<0.01
Enterodiol	0.86 ± 3.09	0.90 ± 1.71	−4.4	<0.01	0.19 (0.06, 0.47)	0.31 (0.11, 0.83)	−38.7	<0.01
Citrus flavonoids	8.71 ± 18.80	6.10 ± 15.40	42.7	0.45	0.83 (0.23, 4.92)	0.78 (0.28, 2.69)	6.4	0.29
Hesperetin	3.11 ± 8.75	1.63 ± 5.01	90.8	0.52	0.13 (0.03, 0.64)	0.14 (0.04, 0.39)	−7.1	0.74
Naringenin	5.60 ± 12.61	4.47 ± 11.8	25.3	0.50	0.66 (0.13, 3.66)	0.57 (0.16, 2.38)	15.8	0.56

[a] Expressed as nmol/mg creatinine.
[b] Expressed as $(\text{mean}_{\text{cases}} - \text{mean}_{\text{controls}})/\text{mean}_{\text{controls}}$ or $(\text{median}_{\text{cases}} - \text{median}_{\text{controls}})/\text{median}_{\text{controls}}$.
[c] From paired *t* tests using log-transformed data.
[d] Include daidzein, genistein, glycitein, O-DMA, dihydrodaidzein, and dihydrogenistein.

justing for body weight. Age at first live birth, history of fibroadenoma, total meat intake, and regular physical activity were adjusted in subsequent analyses because the differences between cases and controls were statistically significant or marginally significant.

Presented in Table 2 are comparisons of the mean and median urinary excretion rates of phytoestrogens between cases and controls. Urinary excretion rate of isoflavonoids was the highest among these three groups of phytoestrogens. The mean and median excretion rates of total and individual parent isoflavonoid (daizein, genistein, and glycitein), particularly glycitein, were substantially lower in cases than controls. The rate of total isoflavonoid metabolites (O-DMA, dihydrodaidzein, and dihydrogenistein) was also lower among cases than controls, although the mean but not median difference was statistically significant. Urinary excretion rates of total and individual mammalian lignans were lower in cases than controls ($P < 0.01$). The mean and median differences between cases and controls were not statistically significant for citrus flavonoids.

Summarized in Table 3 are ORs of breast cancer risk associated with urinary excretion rates of phytoestrogens. After adjusting for potential confounders, the risk of breast cancer decreased with increasing urinary excretion of total isoflavonoids (*P* for trend, 0.04) and total lignans (*P* for trend, <0.01), with ORs of 0.62 (95% CI, 0.39–0.99) and 0.40 (95% CI, 0.24–0.64) for the highest tertile of total isoflavonoid and lignan excretion, respectively. The inverse association was observed for each of the isoflavonoids and lignans included in the study. There was no significant association observed between citrus flavonoids and breast cancer risk. The inverse associations between total isoflavonoid and lignan excretion and breast cancer risk were observed in pre- and postmenopausal women, although the inverse association with lignan excretion was more pronounced in premenopausal women.

In Table 4, the possible joint effects of three classes of phytoestrogens were examined. High excretion rates of both total isoflavonoids and glycitein were consistently related to reduced risks of breast cancer, regardless of the levels of lignans and citrus flavonoids. High excretions of both total isoflavonoids and total lignans were related to an ~70% reduced risk of breast cancer (OR, 0.28; 95% CI, 0.15–0.50). Similar reduction in risk (OR, 0.22; 95% CI, 0.12–0.42) was observed for those who had a high urinary excretion of both glycitein and total lignans. Lignans were associated with a reduced risk of breast cancer, regardless of levels of citrus

Table 3 Adjusted ORs and 95% CIs for the association of breast cancer with urinary excretion of phytoestrogens, Shanghai, 1996–1998

	ORs for urinary phytoestrogen excretion (by tertile)[a]			
	T1	T2	T3	*P* for trend
All subjects (250 pairs)				
Total isoflavonoids[b]	1.00	0.69 (0.44–1.08)	0.62 (0.39–0.99)	0.04
Parent compounds	1.00	0.53 (0.34–0.83)	0.56 (0.35–0.90)	0.01
Daidzein	1.00	0.52 (0.33–0.81)	0.54 (0.34–0.85)	<0.01
Genistein	1.00	0.90 (0.57–1.40)	0.65 (0.41–1.03)	0.07
Glycitein	1.00	0.42 (0.27–0.66)	0.42 (0.25–0.70)	<0.01
Metabolites	1.00	0.57 (0.36–0.89)	0.64 (0.41–1.02)	0.04
O-DMA	1.00	0.64 (0.40–1.01)	0.72 (0.45–1.16)	0.15
Dihydrodaidzein	1.00	0.41 (0.25–0.66)	0.73 (0.47–1.14)	0.08
Dihydrogenistein	1.00	0.55 (0.35–0.87)	0.57 (0.36–0.90)	0.01
Lignans	1.00	0.49 (0.31–0.79)	0.40 (0.24–0.64)	<0.01
Enterolactone	1.00	0.72 (0.46–1.13)	0.42 (0.25–0.69)	<0.01
Enterodiol	1.00	0.70 (0.45–1.10)	0.43 (0.26–0.71)	<0.01
Citrus flavonoids	1.00	0.71 (0.44–1.15)	1.04 (0.66–1.63)	0.86
Hesperetin	1.00	0.46 (0.28–0.75)	0.87 (0.54–1.39)	0.42
Naringenin	1.00	0.76 (0.48–1.19)	1.02 (0.66–1.60)	0.92
Premenopausal women (132 pairs)				
Total isoflavonoids[b]	1.00	0.65 (0.33–1.29)	0.72 (0.36–1.44)	0.33
Lignans	1.00	0.34 (0.16–0.69)	0.24 (0.12–0.50)	<0.01
Citrus flavonoids	1.00	0.73 (0.37–1.41)	1.53 (0.77–3.04)	0.27
Postmenopausal women (118 pairs)				
Total isoflavonoids[b]	1.00	0.76 (0.40–1.46)	0.54 (0.28–1.06)	0.07
Lignans	1.00	0.60 (0.30–1.17)	0.62 (0.31–1.26)	0.16
Citrus flavonoids	1.00	0.75 (0.35–1.61)	0.79 (0.41–1.51)	0.51

[a] Adjusted for age at first live birth, ever diagnosed with fibroadenoma, total meat intake, and physical activity level.
[b] Include daidzein, genistein, glycitein, O-DMA, dihydrodaidzein, and dihydrogenistein.

Table 4 Adjusted ORs and 95% CIs for breast cancer by joint distribution of urinary excretion of isoflavonoids with lignans and citrus flavonoids, Shanghai, 1996–1998

Excretion rate by median	Cases/controls	OR (95% CI)	Cases/controls	OR (95% CI)
Lignans (excretion rate by median)		<4.16		≥4.16
Total isoflavonoids				
<23.09	114/64	1.00 (reference)	38/61	0.32 (0.18–0.56)
≥23.09	65/62	0.52 (0.31–0.88)	33/63	0.28 (0.15–0.50)
Glycitein				
<1.17	97/84	1.00 (reference)	55/41	0.29 (0.16–0.53)
≥1.17	25/39	0.39 (0.22–0.69)	73/86	0.22 (0.12–0.42)
Citrus flavonoids				
<0.78	83/50	1.00 (reference)	38/61	0.31 (0.17–0.57)
≥0.78	96/76	0.75 (0.47–1.22)	33/63	0.38 (0.20–0.72)
Citrus flavonoids (excretion rate by median)		<0.78		≥0.78
Total isoflavonoids				
<23.09	97/84	1.00 (reference)	55/41	1.26 (0.75–2.12)
≥23.09	25/39	0.67 (0.37–1.22)	73/86	0.73 (0.46–1.15)
Glycitein				
<1.17	98/82	1.00 (reference)	61/44	1.25 (0.76–2.07)
≥1.17	24/41	0.53 (0.29–0.98)	67/83	0.60 (0.36–0.99)

[a] Adjusted for age at first live birth, ever diagnosed with fibroadenoma, total meat intake, and physical activity level.

flavonoids. In contrast, citrus flavonoids were not found to be associated with breast cancer risk, regardless of the excretion rates of isoflavonoids and lignans.

Discussion

In this population-based case-control study, we found that breast cancer cases excreted substantially lower levels of urinary isoflavonoids and lignans than did controls, and high excretions of these phytoestrogens were associated with a substantially reduced risk of breast cancer. In particular, women with high excretions of both total isoflavonoids and lignans were associated with a 70% reduced risk of breast cancer. These findings are supported by substantial evidence from ecological studies as well as *in vitro* and *in vivo* experiments (1), suggesting potential cancer-inhibitory effects of these phytoestrogens (1, 21–24).

Evidence from previous analytical epidemiological studies evaluating soyfood intake and breast cancer risk was inconsistent (5, 25, 26). The primary concern was potential measurement error, because none of the food questionnaires used in these studies were formally validated. Using a validated food frequency questionnaire, we found in the Shanghai Breast Cancer Study an inverse association of regular soyfood consumption (at least weekly) with the risk of breast cancer (17). In addition to the level and frequency of phytoestrogen intake, bioavailability of phytoestrogens also depends on the level of metabolism and absorption of these compounds. After high soy protein treatment, a 16- and 15-fold interindividual variation was observed for urinary excretion of total isoflavonoids and lignans, respectively (8). Some metabolites, such as O-DMA, have more striking variation in urinary excretion than parent compounds of soy isoflavones (8). Therefore, urinary excretion of phytoestrogens may be a better measurement of bioavailable phytoestrogens than dietary assessment of soy intake.

The results from this study supported our earlier findings from a small study conducted in the same population (11). The median level of isoflavonoids reported in the current study, however, was higher than that reported previously, because LC/MS, a more sensitive method, was used, and two additional isoflavonoids were included in the current study. Two other earlier, case-control studies also found a substantially reduced risk of breast cancer with urinary excretion of some isoflavonoids and lignans (10, 12). However, the only published cohort study found a weak (20%) and nonsignificantly reduced risk for the highest tertile of genistein excretion and a nonsignificant increased risk for the highest excretion of lignan enterolactone among postmenopausal Dutch women (13). With the exception of our earlier study, the other three published studies were conducted in Western populations with very low soyfood intakes. For example, the median value of urinary genistein in our control groups was two to three times higher than that in the Dutch cohort study (13). Because of a low soy intake in the Western population, an intraindividual variation in urinary excretion of isoflavonoids may be substantial (27, 28), and thus urinary excretion of isoflavonoids may reflect only very recent soyfood intake instead of usual or long-term soyfood consumption. Even in our study population in Shanghai, the correlation at the individual level between usual soy protein intake and urinary isoflavonoid excretion was only modest, although at the group level, a dose-response relationship was observed in both our previous and current studies (9). The medians of urinary excretion rate of isoflavonoids were 5.74 among women eating soyfoods less than once a week, and 18.51, 24.08, and 26.56 with increasing tertile of soy protein intake among those who consumed soyfoods at least once a week. These results support the notion that urinary phytoestrogens are determined not only by intake levels but also by the rate of absorption and metabolism. Also, these results may explain the stronger association for urinary soy biomarkers reported in the current paper than that for usual soy intake reported in our previous paper (17).

Our findings suggested that glycitein may possess stronger protective effects against breast cancer than genistein. Most studies thus far have focused on the biological effects of genistein and daidzein. A recent study found that glycitein induced a three times higher estrogenic response in *in vivo* and a 10-fold lower binding affinity with estrogen receptor *in vitro* compared with genistein (29). A subsequent study suggested that glycitein and daidzein have higher bioavailability than genistein (30). In the Dutch cohort study (13), however, neither glycitein nor daidzein were examined. The reasons for conflicting results of urinary excretion of lignans are not clear. Lignan

intake levels are, in general, higher in the Western population than those in Asian populations (1). However, the urinary excretion rate of lignans in the Dutch cohort study was substantially lower than the rate of isoflavonoids in our study populations (1, 13). The mechanisms by which isoflavonoids and lignans interact with estrogens, estrogen receptors, and related enzymes may be different (1). One recent study found that the determinants of plasma enterolactone concentration include lignan-containing foods and constipation, as well as age and weight (31). Our findings also suggested that the effects of lignans may appear more pronounced among premenopausal women. Therefore, the effects of lignans may be dependent on factors associated with endogenous hormone levels.

Citrus flavonoids, naringenin and hesperetin, are another group of phytoestrogens. In addition to steroid hormone-mediated effects (15), citrus flavonoids also possess other cancer-inhibitory effects, such as antioxidant properties (32, 33), and inhibition of neoplastic transformation on aberrant crypt formation (34, 35). However, no clear association was observed in our study. No study, to our knowledge, has been published thus far to evaluate the association between urinary excretion of citrus flavonoids and breast cancer risk. In contrast to a high and stable intake of soyfoods in our study population, consumption of citrus flavonoids was low and may be affected substantially by seasonal variability in Shanghai. Therefore, urinary excretion of citrus flavonoids may not be a stable biomarker of usual intake of citrus flavonoids in our study population.

A potential concern of this study is that urine samples for cases were collected after initial cancer diagnosis. To minimize potential influence of breast cancer and its sequelae on the levels of urinary analytes, we included in this study only cases whose urine samples were collected before any cancer therapy. Bias, however, could still have occurred, if breast cancer patients had modified substantially their dietary habits after initial cancer diagnosis. Our data show that >90% of cases reported no appreciable dietary change during the time period from initial cancer diagnosis to urine collection. In addition, there is no reason to speculate that cases would have reduced their intake of soyfoods, fruits, and vegetables, particularly because these foods are traditionally believed in Shanghai to be well-balanced foods that are suitable for cancer patients. In our earlier study, we also assayed the urine samples for total nitrogen excretion, a measure of dietary intake of protein, to examine the possibility that our results might be attributable to an overall reduction in food intake among cases after breast cancer initial diagnosis. We found that the medians of total nitrogen excretion were comparable between cases (9.0 mg/mg creatinine) and controls (10.7 mg/mg creatinine), indicating that this bias, if it exists, may not be large. Although the urine samples for all cases were collected before their breast surgery, the urine collected for many cases occurred at the same day (in the morning) of the surgery or only 1–2 days before the surgery. It might be possible that the cases may have modified their dietary intakes as the surgery day approached. To evaluate this possibility, we analyzed data from 44 case-control pairs, in which the urine samples from cases were collected several days before cancer surgery. The analyses generated similar results, although some data did not reach statistical significance because of a small sample size. For example, the mean excretion rate (nmol/mg creatinine) of isoflavonoids were 28.60 for cases and 36.99 for controls, and the mean excretion rates (nmol/mg creatinine) of lignans were 4.43 for cases and 8.30 for controls. The adjusted ORs decreased from 1.00 to 0.63 and 0.63 with increasing excretion of total isoflavonoids and from 1.00 to 0.27 and 0.30 with increasing excretion of lignans.

In conclusion, this case-control study showed that in a population with high soyfood consumption, urinary excretion of isoflavonoids and lignans was substantially and significantly higher in controls than cases. These findings suggest that high bioavailable levels of some phytoestrogens may reduce the risk of breast cancer. Cohort studies are needed to further validate these findings.

Acknowledgments

We thank Drs. Ann Coker and Kim Creek for helpful comments.

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