

# Urinary Excretion of Isoflavonoids and the Risk of Breast Cancer<sup>1</sup>

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## Abstract

**Isoflavonoids are a group of biologically active phytochemicals that humans are exposed to mainly through soy food intake. Because of the similar chemical structure of these compounds and estradiol, it has been hypothesized that isoflavonoids may be related to the risk of breast cancer. Overnight urine samples from 60 incident breast cancer cases and their individually matched controls were assayed for urinary excretion rates of five major isoflavonoids (daidzein, genistein, glycitein, equol, and *O*-desmethylangolensin) and total phenols. These subjects were from a large population-based case-control study conducted in Shanghai, and urine samples from breast cancer cases were collected before any cancer therapy to minimize the potential influence of the disease and its sequelae on study results. Urinary excretion of total phenols and all individual isoflavonoids, particularly glycitein, was substantially lower in breast cancer cases than controls. For total isoflavonoids, the mean excretion was 13.95 nmol/mg creatinine (SD, 20.76 nmol/mg creatinine) for cases and 19.52 nmol/mg creatinine (SD, 25.36 nmol/mg creatinine) for controls (*P* for difference = 0.04). The case-control difference was more evident when median levels of these compounds were compared, with the median excretion of all major isoflavonoids being 50–65% lower in cases than in controls. Individuals in the highest tertile of daidzein, glycitein, and total isoflavonoids had about half the cancer risk of those in the lowest tertile. The adjusted odds ratio for breast cancer was 0.14 (95% confidence interval, 0.02–0.88) for women whose urinary excretion of both phenol and total isoflavonoids was in the upper 50% compared with those in the lower 50%. The results from this study support the hypothesis that a high intake of soy foods may reduce the risk of breast cancer.**

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## Introduction

The incidence rates of breast cancer are substantially lower in China, Japan, and other Asian countries than in the United States and Europe (1). One of the possible explanations for this international difference is that Asian women consume a much higher level of soy foods than their Caucasian counterparts (2–4). Although evidence from ecological studies and laboratory investigations has strongly implicated that soy foods may be protective against breast cancer, only a few epidemiological studies have been published, and the results were inconsistent (2–8). Part of the inconsistency may be due to an inaccuracy in the measurement of the dietary intake levels of these foods. Furthermore, because soy foods are mainly consumed by Asians, it is usually difficult to conduct an epidemiological study to investigate the association of soy foods with breast cancer in non-Asian populations (2, 3, 8).

The potential protective effects of soy foods are largely attributed to the cancer-inhibitory effects of isoflavonoids and possibly other compounds, such as protease inhibitors, saponins, and inositol phosphates (2–4). Isoflavonoids are a group of biologically active phytochemicals that humans are exposed to primarily through soy food intake (2–4, 9). After consumption, the plant isoflavonoids undergo many metabolic conversions by intestinal bacteria, and both the metabolites and parent compounds are absorbed into the blood and then excreted, mainly in the urine (2). Isoflavonoids have a diphenolic structure similar to that of estradiol and have been shown to have a weak estrogenic or antiestrogenic activity in many *in vitro* and *in vivo* systems (2, 3, 10). Another group of phytochemicals with weak estrogenic activity is the lignans, which humans are exposed to mainly through whole grains, seeds, and some fruits and vegetables (2, 3). Because of their influence on estrogen activity, isoflavonoids and lignans are referred to as phytoestrogens (2, 3).

The tumor-inhibitory effect of phytoestrogens has been demonstrated in breast cancer cell lines and in animal models, including mammary tumor models (2, 3, 11). This beneficial effect may be through various mechanisms including the anti-estrogenic, antioxidative, apoptosis-inducing (12), topoisomerase-inhibitory (13), and angiogenesis-inhibitory activities of these compounds (2, 3). Only a few human studies have been performed, and most of them were ecological or cross-sectional, showing that women living in areas with low breast cancer rates or consuming vegetarian diets excrete higher levels of isoflavonoids or lignans than those living in high-risk areas or consuming standard Western diets (2, 3). Recently, a case-control study from Australia reported that a high excretion of some phytoestrogens was associated with a reduced risk of breast cancer (14). However, the intake levels of soy foods in that population were relatively low. In addition, genistein, one of the two major isoflavonoids, was not included in that study because of some technical difficulties in the assay of this compound. Therefore, the Australian study reported results only on two isoflavonoids and three lignans. In this study, we

report an association between the urinary excretion of all major dietary isoflavonoids and breast cancer risk in Chinese women who were studied during 1996 to 1997.

### Materials and Methods

This study is part of the Shanghai Breast Cancer Study, a population-based case-control study conducted among Chinese women in the urban Shanghai area to comprehensively evaluate etiological and prognostic factors for breast cancer. The aim of the study was to recruit all eligible Shanghai residents who were newly diagnosed with breast cancer at the ages of 25–64 years over an 18-month period and a representative sample of women from the general population. Through a rapid case ascertainment system, supplemented by the population-based Shanghai Tumor Registry, 808 incident breast cancer cases were identified by August 1997, and in-person interviews were completed for 746 (92%) of them. The major reasons for nonparticipation were refusal (38 cases; 5%), death before interview (8 cases; 1%), and inability to locate (16 cases; 2%). Data on histopathological diagnosis were collected for all cases.

Controls were randomly selected from the general population and frequency-matched to cases by age using 5-year intervals. The number of controls in each age-specific stratum was determined in advance according to the age distributions of the incident breast cancer cases reported to the Shanghai Tumor Registry from 1990–1993. The Shanghai Resident Registry, which keeps registry cards for all adult residents in urban Shanghai, was used to select controls. For each age-predetermined control, a registry card identifying a potential control in the same 5-year age group was randomly selected. In-person interviews were completed for 649 of the 758 (85.6%) eligible controls identified. A total of 109 potential controls were excluded from the study because of refusal (6%), death or a prior cancer diagnosis (1%), and inability to locate (7%).

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 history, and family history of cancer. All participants were also measured for their current weight, circumferences of the waist and hip, and sitting and standing heights. Among those who completed the interviews and anthropometrics, 1174 (83.5% of cases and 84.9% of controls) donated blood samples, and 1384 (98.7% of cases and 99.8% of controls) donated urine samples. All 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 cancer diagnosis. As a result, blood and urine samples were collected before any cancer therapy from about 70% of cases. The urine samples were collected, and 125 mg of ascorbic acid were added to about 100 ml of urine to prevent the oxidation of labile compounds. Immediately after collection, the samples were placed in portable insulated cases with ice pads (0°C–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 controls who were individually matched to the index cases by age ( $\pm 3$  years), menopausal status (yes, no), and

the date of sample collection ( $\pm 30$  days). To study the relationship between urinary excretion of isoflavonoids and breast cancer, 60 case-control pairs who were recruited into the individually matched study before the summer of 1997 were selected, and their urine samples were assayed for isoflavonoids, phenols, and nitrogen. To eliminate the effect of between-assay variability on the study results, samples for each case-control pair were assayed in the same batch.

HPLC<sup>3</sup> analyses were carried out as described previously (9, 15). Briefly, 2 ml of urine were extracted with 2 ml of diethyl ether after the addition of triethylamine acetate buffer (pH 7.0) and 20  $\mu$ l of flavone (100 ppm in 96% ethanol) as an internal standard and incubated for 1 h at 37°C with 10  $\mu$ l of  $\beta$ -glucuronidase and 10  $\mu$ l of arylsulfatase. The combined ether fractions were evaporated to dryness under a stream of nitrogen and redissolved in 100  $\mu$ l of methanol and 100  $\mu$ l of 0.2 M sodium acetate buffer (pH 4.0). Samples were analyzed immediately by injecting 20  $\mu$ l into the HPLC system or stored at –20°C until analyzed. HPLC analyses were carried out using a NovaPak C18 (150  $\times$  3.9 mm; inner diameter, 4  $\mu$ m) reversed-phase column (Waters, Milford, MA). Elution was performed at a flow rate of 0.8 ml/min with the following linear gradient: A, acetic acid/water (10:90, v/v); B, methanol/acetonitrile/dichloromethane (10:5:1, v/v/v); B in A (v/v), 5% for 5 min, from 5 to 45% in 20 min, from 45 to 70% in 6 min, and from 70 to 5% in 3 min, with equilibration for 15 min before subsequent injection. Analyses were monitored simultaneously by photodiode array detection at 260 and 280 nm during the entire HPLC run. Observed signals were scanned between 190 and 400 nm for identification purposes and quantitated using peak areas after calibration with authentic standards (16).

Urinary phenol excretion, a potential surrogate marker of fruit and vegetable intake (17, 18), was included in this study mainly to evaluate whether the association of isoflavonoids with breast cancer, if it exists, is independent of other dietary phytochemicals. Urine (50  $\mu$ l) was mixed with 50  $\mu$ l of 95% ethanol followed by vortex mixing for 2 min and centrifugation for 5 min at 1,100  $\times$  g. Clear supernatant (20 and 40  $\mu$ l) was diluted with distilled water to 2.0 ml followed by thorough mixing with 0.2 ml of Folin-Ciocalteu Phenol Reagent (Sigma, St. Louis, MO; catalogue number F9252; Ref. 19) and incubation for 3 min at room temperature. After the addition of 0.4 ml of saturated aqueous sodium carbonate solution (approximately 35%) and 1.4 ml of distilled water, the mixture was incubated for exactly 1 h at room temperature before reading the absorbance at 725 nm against a reagent blank. As a reference, solutions with a final concentration of 10 and 20  $\mu$ M aqueous quercetin were measured in the same way. Concentrations of quercetin stock solutions in methanol were determined with absorbance readings at 373 nm using 20,892 as the molar absorptivity (20). Final values were expressed as quercetin equivalents in micromolar concentrations that were adjusted to creatinine levels. Mean coefficients of variation for intra- and interassay variability were found to be 5.4 and 8.8%, respectively.

Total nitrogen excretion was used in the study to compare the overall dietary protein intake between cases and controls for a few days before urine collection (14, 21). This was measured with an enzymatic kit (Sigma; catalogue number 66-20) by the urease/glutamate-dehydrogenase-catalyzed reactions of urea and ammonia. Coefficients of variation for intra- and interassay

<sup>3</sup> The abbreviations used are: HPLC, high-performance liquid chromatography; OR, odds ratio; CI, confidence interval.

Table 1 Comparison of cases and controls by selected demographic and risk factors, Shanghai, 1996–1997

Characteristic	Cases (n = 60)	Controls (n = 60)	P <sup>a</sup>
Demographic factors			
Age (yr) <sup>b</sup>	46 (41, 49)	46 (41, 50)	0.96
≥High school education (%)	35.0	35.0	1.00
Nondietary risk factors			
First-degree relative with breast cancer (%)	3.3	0	0.37
Ever diagnosed with benign breast disease (%)	35.0	30.0	0.68
Body mass index <sup>b</sup>	23 (21, 25)	23 (20, 25)	0.55
No leisure physical activity (%)	81.7	65.0	0.13
Age at menarche <sup>b</sup>	15 (14, 16)	15 (14, 16)	0.77
Age at menopause (yr) <sup>b,c</sup>	50 (45, 51)	48 (45, 49)	0.69
Parity <sup>b</sup>	2 (1, 3)	2 (2, 3)	0.83
Age at first pregnancy (yr) <sup>b,d</sup>	28 (25, 30)	26 (24, 28)	0.03
Nutritional factors			
Total energy intake (kcal/day) <sup>b</sup>	2301 (1937, 2593)	2257 (2210, 2732)	0.55
Total fat intake (g/day) <sup>b</sup>	70 (58, 82)	75 (60, 93)	0.86
Total fruit and vegetable intake (g/day) <sup>b</sup>	337 (223, 580)	363 (260, 521)	0.67
Protein intake from soyfoods (g/day) <sup>b</sup>	8.7 (5.2, 13.3)	8.7 (4.6, 15.4)	0.80

<sup>a</sup> P<sub>s</sub> were from the  $\chi^2$  test (for categorical variables) or Wilcoxon signed rank test (for continuous variables).

<sup>b</sup> Median (25<sup>th</sup>, 75<sup>th</sup> percentile) values are presented.

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

<sup>d</sup> Among ever pregnant women.

variability were found to vary between 0.6 and 2.4% and 2.6 and 4.7%, respectively. Urinary creatinine concentrations were determined by a test kit based on the Jaffe reaction (Sigma; catalogue number 555; Ref. 22). Mean coefficients of variation for intra- and interassay variability were found to be 4.1 and 6.7%, respectively.

Isoflavonoid and phenol excretion in urine were expressed in nanomole (isoflavonoids) or micromole quercetin equivalents (phenols) per milligram of creatinine by adjusting isoflavonoid and phenol concentrations for urinary creatinine levels. Because the data were skewed to a high value, log-transformed data were used in the paired Student *t* tests to compare the mean differences between cases and controls (23). The Wilcoxon signed rank tests were also used for comparisons of the median difference between cases and controls. To evaluate the potential dose-response relation between urinary excretion rates of isoflavonoids and phenols 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 tertiles were derived using conditional logistic regression compared to the lowest tertile group (24). Multivariate analyses were performed to adjust for potential confounding variables. Tests for trend across the tertile were performed in logistic regressions by assigning the score *j* to the *j*th level of the variable selected. All statistical analyses were based on two-tailed probability.

## Results

The distributions of selected demographic characteristics, nutritional factors, and established major risk factors for breast cancer are shown in Table 1. Cases and controls were comparable in age and education. More breast cancer cases than controls reported having a family history of breast cancer, ever being diagnosed with a benign breast disease, and having no leisure physical activity. The median ages for menopause and first pregnancy were higher in cases than in controls. Although not statistically significant due to the small sample size, these findings were consistent with those reported previously in other populations (25). There is no substantial difference between

cases and controls in the intake levels of total energy, fat, fruits and vegetables, and soy protein. Therefore, an adjustment was made only for age at first pregnancy and physical activity in multivariate analyses.

Table 2 compares the mean and median excretion rates of isoflavonoids and phenols between cases and controls. The mean excretion rates of total isoflavonoids and all individual isoflavonoids were lower in cases than controls, although the differences were statistically significant only for glycitein ( $P < 0.01$ ) and total isoflavonoids ( $P = 0.04$ ). The median comparisons revealed even more striking case-control differences in the isoflavonoid excretion rates than did mean comparisons. Again, the differences were statistically significant only for glycitein and total isoflavonoids. The phenol excretion rates were lower in cases than controls, but the difference was not statistically significant.

Table 3 presents ORs of breast cancer risk associated with urinary excretion rates of phenols and major isoflavonoids. Equol and *O*-desmethylangolensin were not analyzed separately because their extremely low levels in urine samples make the grouping of cases and controls difficult; they were included, along with other isoflavonoids, in the estimate of total isoflavonoids. The risks of breast cancer were reduced with high excretion of isoflavonoids and phenols, although the tests for dose-response relationships were statistically significant only for glycitein and total isoflavonoids. After an adjustment for potential confounding variables, the inverse associations persisted, but the trend tests were no longer statistically significant.

To assess whether the effects of isoflavonoids are independent of other phenolic compounds as measured by urinary phenol excretion, stratified analyses were performed (Table 4). Due to the small number of study subjects, cases and controls were categorized into only two groups according to the median distribution of the control group. High excretions of both total isoflavonoids and glycitein were consistently related to reduced risks of breast cancer, regardless of the levels of phenols. In particular, high excretions of both total isoflavonoids and phenols were related to an 85% reduction in the risk of breast cancer (OR, 0.14; 95% CI, 0.02–0.88). A similar reduction in

Table 2 Comparison of urinary excretion of isoflavonoids and phenols between breast cancer cases and controls, Shanghai, 1996–1997

	Mean $\pm$ SD		% of difference	<i>P</i> from paired <i>t</i> test <sup>a</sup>	Median (25 <sup>th</sup> , 75 <sup>th</sup> percentile)		% of difference <sup>b</sup>	<i>P</i> from Wilcoxon test
	Cases	Controls			Cases	Controls		
Total isoflavonoids <sup>c</sup>	13.95 $\pm$ 20.76	19.52 $\pm$ 25.36	-28.5	0.04	4.41 (1.32,17.5)	10.96 (3.80,24.16)	-59.8	0.05
Daidzein <sup>c</sup>	7.75 $\pm$ 12.34	9.19 $\pm$ 12.64	-15.7	0.10	2.15 (0.75,9.22)	4.50 (2.26,12.12)	-52.2	0.08
Genistein <sup>c</sup>	3.57 $\pm$ 5.92	4.06 $\pm$ 5.19	-12.1	0.07	0.76 (0.28,4.33)	2.17 (0.64, 4.94)	-65.0	0.09
Glycitein <sup>c</sup>	1.27 $\pm$ 1.82	2.54 $\pm$ 3.28	-50.0	<0.01	0.45 (0.1,69)	1.36 (0.33, 3.03)	-66.9	0.02
Equol <sup>c</sup>	0.41 $\pm$ 1.86	0.83 $\pm$ 2.38	-50.6	0.11	0 (0, 0)	0 (0, 0)		0.19
O-DMA <sup>c, d</sup>	0.94 $\pm$ 2.18	2.90 $\pm$ 10.01	-67.6	0.28	0 (0, 1.11)	0 (0, 1.33)		0.33
Phenols <sup>e</sup>	6.95 $\pm$ 4.85	10.13 $\pm$ 14.70	-31.4	0.22	5.65 (3.32,8.68)	6.87 (3.53,10.50)	-17.8	0.16

<sup>a</sup> From paired *t* tests using log-transformed data.

<sup>b</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>c</sup> Expressed as nmol/mg creatinine.

<sup>d</sup> O-DMA, *O*-desmethylangolensin.

<sup>e</sup> Expressed as  $\mu$ mol quercetin equivalents/mg creatinine.

Table 3 ORs and 95% CIs for the association of breast cancer with urinary excretion of isoflavonoids and phenols, Shanghai, 1996–1997

Urinary excretion (by tertile) <sup>a, b</sup>	No. of cases	No. of controls	Unadjusted OR (95% CI)	Adjusted OR (95% CI) <sup>c</sup>
<b>Total isoflavonoids</b>				
<5.58	35	20	1.00	1.00
5.58–18.65	11	20	0.35 (0.14–0.89)	0.50 (0.18–1.36)
$\geq$ 18.66	14	20	0.45 (0.19–1.10)	0.50 (0.19–1.31)
			<i>P</i> = 0.04	<i>P</i> = 0.11
<b>Daidzein</b>				
<2.81	34	20	1.00	1.00
2.81–7.59	9	20	0.35 (0.15–0.84)	0.35 (0.14–0.93)
$\geq$ 7.61	17	20	0.54 (0.24–1.24)	0.54 (0.22–1.32)
			<i>P</i> = 0.10	<i>P</i> = 0.12
<b>Genistein</b>				
<0.98	32	20	1.00	1.00
0.99–4.08	11	20	0.43 (0.19–1.01)	0.47 (0.19–1.14)
$\geq$ 4.09	17	20	0.59 (0.25–1.43)	0.70 (0.27–1.84)
			<i>P</i> = 0.12	<i>P</i> = 0.27
<b>Glycitein</b>				
<0.63	35	20	1.00	1.00
0.63–2.14	13	20	0.43 (0.18–1.04)	0.57 (0.22–1.46)
$\geq$ 2.15	12	20	0.36 (0.14–0.94)	0.41 (0.15–1.11)
			<i>P</i> = 0.02	<i>P</i> = 0.06
<b>Phenols</b>				
<4.4	22	21	1.00	1.00
4.4–9.6	24	20	1.12 (0.42–3.02)	1.55 (0.50–4.75)
$\geq$ 9.7	14	19	0.66 (0.23–1.93)	0.62 (0.19–2.00)
			<i>P</i> = 0.43	<i>P</i> = 0.38

<sup>a</sup> Cases and controls were categorized according to the closest tertile level of controls.

<sup>b</sup> Expressed as nmol/mg creatinine for isoflavonoids and  $\mu$ mol quercetin equivalents/mg creatinine for phenols.

<sup>c</sup> Adjusted for age at first pregnancy and physical activity levels.

risk (OR, 0.14; 95% CI, 0.03–0.79) was observed for those who had a high urinary excretion of both glycitein and phenols.

## Discussion

In this population-based case-control study, we found that women newly diagnosed with breast cancer excreted substantially lower levels of urinary isoflavonoids and phenols than controls, and high excretions of these compounds were associated with a substantially reduced risk of breast cancer. In particular, high excretions of both phenols and total isoflavonoids were associated with an 85% statistically significant reduced risk of breast cancer. Although some of the results from

Table 4 Adjusted ORs<sup>a</sup> and 95% CIs for breast cancer by joint distribution of urinary excretion of phenols and selected isoflavonoids

Isoflavonoids (nmol/mg creatinine, by median)	Phenol ( $\mu$ mol/mg creatinine, by median)			
	<6.87		$\geq$ 6.87	
	No. of cases/controls	OR (95% CI)	No. of cases/controls	OR (95% CI)
<b>Total isoflavonoids</b>				
<10.96	22/14	1.00 (reference)	16/16	0.65 (0.18–2.37)
$\geq$ 10.86	15/16	0.89 (0.31–2.57)	7/14	0.14 (0.02–0.88)
<b>Glycitein</b>				
<1.36	23/12	1.00 (reference)	18/18	0.46 (0.12–1.79)
$\geq$ 1.36	14/18	0.56 (0.18–1.71)	5/12	0.14 (0.03–0.79)

<sup>a</sup> Adjusted for age at first pregnancy and physical activity levels.

this study were not statistically significant (perhaps due to a small sample size), they were remarkably consistent internally, suggesting that our findings may not be due to chance.

Our findings for the inverse association between isoflavonoid excretion and breast cancer risk are supported by a large body of evidence from *in vitro* and animal studies (2–4). Isoflavonoids or soy products have been repeatedly shown to inhibit the growth of human breast cancer cells and the formation of animal tumors, including mammary tumors (2, 3, 11, 18, 26). These beneficial anticancer effects may occur through various mechanisms at many different levels. Isoflavonoids can compete with endogenous estrogens in the binding of estrogen receptors and nuclear estrogen-binding sites, reducing the hormonal effect of endogenous estrogens that are much more potent than isoflavonoids (2–4). Some isoflavonoids have been shown to stimulate the synthesis of sex hormone-binding globulin, decreasing blood levels of free estrogens that are more available to the target tissues (2–4). Certain isoflavonoids may inhibit important steroid biosynthetic enzymes, including aromatase and 17 $\beta$ -hydroxysteroid dehydrogenase I, thus affecting the level of circulating estrogens (2–4). In addition to these antiestrogenic effects, isoflavonoids have also been found to have inhibitory effects on angiogenesis, apoptosis, and tumor invasion as well as antioxidative, antiproliferative, and apoptosis-inducing activities (2, 3, 27, 28). These activities, along with the antiestrogenic activities of isoflavonoids, make these compounds very promising candidates to act as dietary chemopreventive agents, particularly for breast cancer and other hormone-related malignancies.

Despite strong evidence from animal studies and *in vitro* experiments, studies in humans have been few and inconsistent

(2, 3, 5–8). Most earlier studies on urinary isoflavonoids and breast cancer were ecological or cross-sectional studies (2, 3). Although most of these studies have provided some supports for a potential role of isoflavonoids and soy foods in the prevention of breast cancer, the inherent limitations of these types of studies have limited their ability to draw any etiological conclusion (2, 3). Three recent case-control studies have focused on the evaluation of the association between soy food intake and breast cancer risk (5–7). All three studies were conducted in Asian populations, with two of them reporting an inverse association of soy food intake with the risk of breast cancer (5, 7). Several cohort studies have also shown a reduced risk of breast cancer associated with an intake of certain soy foods or legumes, although the information on soy consumption was very limited in those studies (8, 29, 30).

The results from short-term soy intervention studies were also inconsistent, with one study reporting decreased levels of serum estrogens after soy supplementation (31), and other studies reporting no change (32) or even an increase (33) in estrogen levels after soy supplements. Soy supplementation was also found to decrease the plasma levels of luteinizing hormone and follicle-stimulating hormone and increase the length of the menstrual cycle (31, 33). Of note is that in the study by Cassidy *et al.* (33), soy protein, but not soy protein from which the phytoestrogens were removed, increased the length of the menstrual cycle in premenopausal women, suggesting that the effect may be due to isoflavonoid phytoestrogens alone or in combination with soy protein rather than other soy constituents. In a recent study among premenopausal Japanese women, serum levels of estradiol were found to be lower among those whose soy food intake levels were high (34).

Although urinary equol excretion was not found to differ between breast cancer patients and controls (35), a hospital-based case-control study conducted in Australia by Ingram *et al.* (14) reported very recently that the risks of breast cancer were decreased with increasing excretion levels of several urinary phytoestrogens, including daidzein and equol, providing perhaps the most direct evidence thus far for a potentially protective effect of these compounds in human breast cancer (2, 3, 14). Because of a low level soy food intake in that study population and some difficulties in laboratory assays, the Australian study reported results only on daidzein and equol. Compared to the Australian study, our study, a population-based case-control study conducted among Chinese women, showed a higher soy intake, resulting in a 5–10 times higher urinary isoflavonoid excretion based on the assumption of a urinary creatinine excretion of 1 g daily. In addition, our study reported results on all major soy isoflavonoids and their metabolites in relation to breast cancer risk. Our results were consistent with those from the Australian study, providing additional evidence that isoflavonoids may be promising phytochemicals in the prevention of breast cancer in populations with high soy consumption.

The primary concern of this study is that postdiagnostic urine samples were used in the assays of urinary isoflavonoids for breast cancer cases. However, to our knowledge, virtually no existing cohort studies have stored urine samples that can be used to investigate the association of urinary isoflavonoid excretion and breast cancer in a prospective manner. To minimize the potential influence of breast cancer and its sequelae on the levels of urinary analytes, we attempted to collect urine samples from cases as soon as possible after their initial cancer diagnosis. As a result, urine samples from all cases included in this study were collected before any cancer therapy. However, bias could still have occurred if breast cancer patients had

substantially changed their dietary habits after cancer diagnosis, particularly because the urinary isoflavonoids primarily reflect the recent intake of soy foods (2, 9, 36). Our data show that over 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 the cases would have reduced their intake of tofu and other soy foods, particularly because these foods are traditionally believed in Shanghai to be well-balanced foods that are suitable for cancer patients. We also assayed the urine samples for total nitrogen excretion, a measure of dietary intake of protein (37), to examine the possibility that our results might be due to an overall reduction in food intake among cases after breast cancer 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 urinary excretion of isoflavonoids may be a more direct measure of bioavailability of these phytochemicals than dietary assessment, it primarily reflects the intake levels of soy foods over the 24–96-h period before collection (2, 9, 36). Therefore, recent diet may have a major impact on the levels of urinary isoflavonoids; thus, there may be a large intraperson variability of these compounds in urine, a situation that is not optimal for epidemiological studies. The consumption of soy foods, however, is a personal dietary preference, and the intake levels for most individuals are likely to be relatively stable over time, particularly in Shanghai, an area with abundant supplies of soy foods. Indeed, cross-sectional studies among Chinese in Singapore (15) and among Hawaii's multiethnic population (38) found dose-dependent associations between soy intake assessed using a food frequency questionnaire and the major urinary isoflavonoids or the sum of major isoflavonoids obtained from a spot urine about 10–12 months later. A similar association was found in our population (39). These results suggest that urinary isoflavonoids can be used as a measure of soy consumption in epidemiological studies of diet-disease associations.

In summary, despite a small sample size, this population-based study showed that urinary excretion of isoflavonoids was substantially and statistically significantly lower in breast cancer patients than in controls in a population with generally high soy consumption. The findings are biologically plausible and are consistent with results from recent *in vitro*, animal, and epidemiological studies, suggesting a potential beneficial effect of soy foods and isoflavonoids in the prevention of breast cancer.

## References

- Parkin, D. M., Pisani, P., and Ferlay, J. Estimates of the worldwide incidence of eighteen major cancers in 1985. *Int. J. Cancer*, 54: 594–606, 1993.
- Adlercreutz, H., and Mazur, W. Phyto-oestrogens and Western diseases. *Ann. Med.*, 29: 95–120, 1997.
- Horn-Ross, P. Phytoestrogens, body composition, and breast cancer. *Cancer Causes Control*, 6: 567–573, 1995.
- Messina, M., Barnes, S., and Setchell, K. D. Phyto-oestrogens and breast cancer. *Lancet*, 350: 971–972, 1997.
- Lee, H. P., Gourley, L., Duffy, S. W., Esteve, J., Lee, J., and Day, N. E. Dietary effects on breast-cancer risk in Singapore. *Lancet*, 337: 1197–1200, 1991.
- Yuan, J. M., Wang, Q. S., Ross, R. K., Henderson, B. E., and Yu, M. C. Diet and breast cancer in Shanghai and Tianjin, China. *Br. J. Cancer*, 71: 1353–1358, 1995.
- Wu, A. H., Ziegler, R. G., Horn-Ross, P. L., Nomura, A. M. Y., West, D. W., Kolonel, L. N., Rosenthal, J. F., Hoover, R. N., and Pike, M. C. Tofu and risk of breast cancer in Asian-Americans. *Cancer Epidemiol. Biomark. Prev.*, 5: 901–906, 1996.

8. Greenstein, J., Kushi, L., Zheng, W., Fee, R., Campbell, D., Sellers, T., and Folsom, A. Risk of breast cancer associated with intake of specific foods and food groups. *Am. J. Epidemiol.*, *143* (Suppl.): S36, 1996.
9. Franke, A. A., and Custer, L. J. High-performance liquid chromatography assay of isoflavonoids and coumestrol from human urine. *J. Chromatogr. B.*, *662*: 47–60, 1994.
10. Makelä, S., Santti, R., Salo, L., and McLachlan, J. A. Phytoestrogens are partial estrogen agonists in the male mouse. *Environ. Health Perspect.*, *103* (Suppl 7): 123–127, 1997.
11. Lamartiniere, C. A., Moore, J., Holland, M., and Barnes, S. Neonatal genistein chemoprevents mammary cancer. *Proc. Soc. Exp. Biol. Med.*, *8*: 120–123, 1995.
12. Kyle, E., Neckers, L., Takimoto, C., Curt, G., and Bergan, R. Genistein-induced apoptosis of prostate cancer cells is preceded by a specific decrease in focal adhesion kinase activity. *Mol. Pharmacol.*, *51*: 193–200, 1997.
13. Constantinou, A., and Huberman, E. Genistein as an inducer of tumor cell differentiation: possible mechanisms of action. *Proc. Soc. Exp. Biol. Med.*, *208*: 109–115, 1995.
14. Ingram, D., Sanders, K., Kolybaba, M., and Lopez, D. Case-control study of phyto-oestrogens and breast cancer. *Lancet*, *350*: 990–994, 1997.
15. Seow, A., Shi, C. Y., Franke, A. A., Hankin, J. H., Lee, H. P., and Yu, M. C. Isoflavonoid levels in spot urine are associated with frequency of dietary soy intake in a population-based sample of middle-aged and older Chinese in Singapore. *Cancer Epidemiol. Biomark. Prev.*, *7*: 135–140, 1998.
16. Franke, A. A., Custer, L. J., Cerna, C. M., and Narala, K. K. Quantitation of phytoestrogens in legumes by HPLC. *J. Agric. Food Chem.*, *42*: 1905–1913, 1994.
17. Malavelle, C., Hautefeuille, A., Pignatelli, B., Talaska, G., Vines, P., and Bartsch, H. Antimutagenic dietary phenolics as antigenotoxic substances in urothelium of smokers. *Mutat. Res.*, *402*: 219–214, 1998.
18. Malaveille, C., Hautefeuille, A., Pignatelli, B., Talaska, G., Vineis, P., and Bartsch, H. Dietary phenolics as anti-mutagens and inhibitors of tobacco-related DNA adduction in the urothelium of smokers. *Carcinogenesis (Lond.)*, *17*: 2193–2200, 1996.
19. Singleton, V. L., and Rossi, J. Colorimetry of total phenols with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticult.*, *16*: 144–158, 1965.
20. Jurd, L. Spectral properties of flavonoid compounds. In: T. A. Glissmann (ed.), *The Chemistry of Flavonoid Compounds*, pp. 107–155. New York: Mac-Millan, 1962.
21. Burge, J. C., Choban, P., McKnight, T., Kyler, M. M. K., and Flancbaum, L. Urinary ammonia plus urinary urea nitrogen as an estimate of total urinary nitrogen in patients receiving parenteral nutrition support. *J. Parenter. Enteral Nutr.*, *17*: 529–531, 1992.
22. Jaffe, M. Über den Niederschlag, welchen Picrinsäure in normalem Harn erzeugt und über eine neue Reaktion des Kreatinins. *Hoppe-Seyler's Z. Physiol. Chem.*, *10*: 391–398, 1886.
23. Armitage, P., and Berry, G. *Statistical Methods in Medical Research*, 2nd ed. Oxford: Blackwell Scientific Publications, 1988.
24. Breslow, N. E., and Day, N. E. *Statistical Methods in Cancer Research: Analysis of Case-Control Studies*. Lyon, France: IARC, 1980.
25. Henderson, B. E., Pike, M. C., Bernstein, L., and Ross, R. K. Breast Cancer. In: D. Schottenfeld and J. F. Fraumeni, Jr. (eds.), *Cancer Epidemiology and Prevention*, 2nd ed., pp. 1022–1039. New York: Oxford University Press, 1996.
26. Murrill, W. B., Brown, N. M., Zhang, J. X., Manzillo, P. A., Barnes, S., and Lamartiniere, C. A. Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. *Carcinogenesis (Lond.)*, *17*: 1451–1457, 1996.
27. Hirano, T., Oka, K., and Akiba, M. Antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast carcinoma cell line, ZR-75-1. *Res. Comm. Chem. Pathol. Pharmacol.*, *64*: 69–78, 1989.
28. Uckun, F. M., Evans, E., Forsyth, C. J., Waddick, K. G., Ahlgren, T. L., Chelstrom, L. M., Burkhardt, A., Bolen, J., and Myers, D. E. Biotherapy of B-cell precursor leukemia by targeting genistein to CD19-associated tyrosine kinases. *Science (Washington DC)*, *267*: 886–891, 1995.
29. Nomura, A., Henderson, B. E., and Lee, J. Breast cancer and diet among the Japanese in Hawaii. *Am. J. Clin. Nutr.*, *31*: 2020–2025, 1978.
30. Hirayama, T. A large scale cohort study on cancer risk by diet –with special reference to the risk reducing effects of green-yellow vegetable consumption. In: Y. Hayashi, M. Nagao, and T. Sugimura, (eds.), *Diet, Nutrition, and Cancer*, pp. 41–53. Utrecht, the Netherlands: Tokyo Scientific Press, 1986.
31. Lu, L. J. W., Anderson, K. E., Grady, J. J., and Nagamani, M. Effects of soy consumption for one month on steroid hormones in premenopausal women: implications for breast cancer risk reduction. *Cancer Epidemiol. Biomark. Prev.*, *5*: 63–70, 1996.
32. Baird, D. D., Umbach, D. M., Lansdell, L., Hughes, C. L., Setchell, K. D. R., Weinberg, C. R., Haney, A. F., Wilcox, A. J., and McLachlan, J. A. Dietary intervention study to assess estrogenicity of dietary soy among postmenopausal women. *J. Clin. Endocrinol. Metab.*, *80*: 1685–1690, 1995.
33. Cassidy, A., Bingham, S., and Setchell, K. D. R. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *Am. J. Clin. Nutr.*, *60*: 333–340, 1994.
34. Nagata, C., Kabuto, M., Kurisu, Y., and Shimizu, H. Decreased serum estradiol concentration associated with high dietary intake of soy products in premenopausal Japanese women. *Nutr. Cancer*, *29*: 228–233, 1997.
35. Adlercreutz, H., Fotsis, T., Heikkinen, R., Dwyer, J. T., Woods, M., Goldin, B. R., and Gorbach, S. L. Excretion of the lignans enterolactone and enteroidiol and of equol in omnivorous and vegetarian postmenopausal women and in women with breast cancer. *Lancet*, *2*: 1295–1299, 1982.
36. Kelly, G. E., Joannou, G. E., Reeder, A. Y., Nelson, C., and Waring, M. A. The variable metabolic response to dietary isoflavones in humans. *Proc. Soc. Exp. Biol. Med.*, *208*: 40–43, 1995.
37. Huse, D. M., Nelson, R. A., Broines, E. R., and Hodgson, P. A. Urinary nitrogen excretion as object measure of dietary intake. *Am. J. Clin. Nutr.*, *27*: 771–773, 1974.
38. Maskarinec, G., Singh, S., Meng, L., and Franke, A. A. Dietary soy intake and urinary isoflavone excretion among women from a multi-ethnic population. *Cancer Epidemiol. Biomark. Prev.*, *7*: 613–619, 1998.
39. Chen, Z., Zheng, W., Custer, L. J., Dai, Q., Shu, X. O., Jin, F., and Franke, A. A. Usual dietary consumption of soy foods and its correlation with the excretion rate of isoflavonoids in overnight urine samples among Chinese women in Shanghai. *Nutr. Cancer*, in press, 1999.