Phytoestrogen Concentrations in Serum and Spot Urine as Biomarkers for Dietary Phytoestrogen Intake and Their Relation to Breast Cancer Risk in European Prospective Investigation of Cancer and Nutrition-Norfolk

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Abstract

Subjects of this study consisted of 333 women (aged 45–75 years) drawn from a large United Kingdom prospective study of diet and cancer, the European Prospective Investigation of Cancer and Nutrition-Norfolk study. Using newly developed gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry methods incorporating triply ^{13}C-labeled standards, seven phytoestrogens (daidzein, genistein, glycitein, *O*-desmethylangolensin, equol, enterodiol, and enterolactone) were measured in 114 spot urines and 97 available serum samples from women who later developed breast cancer. Results were compared with those from 219 urines and 187 serum samples from healthy controls matched by age and date of recruitment. Dietary levels were low, but even so, mean serum levels of phytoestrogens were up to 600 times greater than postmenopausal estradiol levels. Phytoestrogen concentrations in spot urine (adjusted for urinary creatinine) correlated strongly with that in serum, with Pearson correlation coefficients > 0.8. There were significant relationships ($P < 0.02$) between both urinary and serum concentrations of isoflavones across increasing tertiles of dietary intakes. Urinary enterodiol and enterolactone and serum enterolactone were significantly correlated with dietary fiber intake ($r = 0.13–0.29$). Exposure to all isoflavones was associated with increased breast cancer risk, significantly so for equol and daidzein. For a doubling of levels, odds ratios increased by 20–45% [$\log_2$ odds ratio = 1.34 (1.06–1.70; $P = 0.013$) for urine equol, 1.46 (1.05–2.02; $P = 0.024$) for serum equol, and 1.22 (1.01–1.48; $P = 0.044$) for serum daidzein]. These estimates of risk are similar to those established for estrogens and androgens in postmenopausal breast cancer but need confirmation in larger studies. (Cancer Epidemiol Biomarkers Prev 2004;13(5): 698–708)

Introduction

Phytoestrogens are naturally occurring diphenolic compounds from plants that are structurally similar to the hormone 17β-estradiol. They are absorbed from food, circulate in the bloodstream, and are excreted in urine. Of the several groups of phytoestrogens, the isoflavones include glycitein, daidzein, and genistein, which are found naturally in foods including legumes and especially soy, and their metabolites equol and *O*-desmethylangolensin (O-DMA) (1). The lignans are a separate group and include enterolactone and enterodiol, derived from colonic microbial fermentation of matairesinol and secoisolariciresinol found in a wide variety of plant foods. Possible anticarcinogenic effects of these compounds in breast cancer are attributed to their capability to bind to estrogen receptors α and β, and they may exert antiestrogenic effects through inhibitory binding (2–6). They may also act as antioxidants (7, 8) or inhibit several key enzymes such as tyrosine kinase (9) and DNA topoisomerase (10). However, phytoestrogens are weak estrogens and also inhibit sulfotransferases involved in the removal of endogenous estradiol (11). In *in vivo* assays, genistein and equol are more estrogenic than daidzein and the lignans, with uterotrophic potencies in the order of 0.001 times the activity of estradiol, although there are interspecies differences in susceptibility (1). It is well established that circulating endogenous sex hormones increase the risk of breast cancer in women, even at low average levels of 50 pmol/l estradiol (12).

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Epidemiological studies investigating the role of phytoestrogens in cancer have produced inconclusive results. Case-control studies point to the protective effect of phytoestrogen intake on breast cancer (13–18), but there is evidence of an estrogenic effect on the breast after soy supplementation (1, 19). In addition, six of seven prospective studies published on phytoestrogen and breast cancer risk showed nonsignificant results (20–26). The study by Nomura *et al.* (20) used diets reported by husbands of women with breast cancer as a surrogate measure of diets of these women. The studies by Greenstein *et al.* (21), den Tonkelaar *et al.* (22), and Horn-Ross *et al.* (23) were done in Western populations with low phytoestrogen consumption, which could limit their abilities to detect significant associations. The dietary questionnaires used in the Japanese studies by Key *et al.* (24) and Hirayama (25) had not been validated for phytoestrogen consumption, and exposure misclassification may mask any true diet-disease associations. Only one study in Japanese women found that consumption of miso soup and isoflavones, but not of soy foods, was inversely associated with breast cancer risk (26).

Large-scale epidemiological studies have been limited by difficulties in quantifying phytoestrogen intake. This is attributable to the paucity of data on phytoestrogen concentrations in food and dietary intakes, especially for the lignans. The isolation and analysis of phytoestrogens in foods is difficult given the range of matrices in which these phytoestrogens are found and the substantial variation according to variety, crop, season, location, and processing methods (27–31). Food composition databases of phytoestrogen content are expensive and technically difficult to establish and are often unavailable in most epidemiological studies. Thus, measuring lignans and isoflavones in biological samples such as urine and serum offers an attractive alternative approach to estimating human exposure to these compounds, provided that suitable analytical methods are used.

Several studies (14, 18, 22, 32–34) have used urine and serum levels as a biomarker of phytoestrogen intake, but validity studies of this procedure have assessed subjects on a controlled diet supplemented with increasing dose of soy protein (35) or have been carried out on populations with high habitual isoflavones or soy intakes (36–41). Among the few studies that had reported correlation information between dietary and urinary or serum phytoestrogens in Western populations, three studies (42–44) related soy intake to plasma phytoestrogen concentrations, while two studies (45, 46) related dietary to urinary phytoestrogens. Notably, almost all studies that investigated urinary phytoestrogen (except one; Ref. 37) have used extensive urinary collections ranging from overnight to as long as 72 h (36, 38–41, 46, 47). The utility of phytoestrogen concentrations in untimed spot urine as biomarkers of dietary phytoestrogen intakes in large-scale epidemiological studies in Western populations remains largely unknown. In addition, in response to soy challenge, only about one-third of individuals are thought to produce the urinary metabolite equol (1), which has been proposed to be the most important in explaining the mechanism of action of isoflavones in disease prevention and treatment (48). However, little is known of the relation between blood and urine levels in individuals consuming low amounts.

Previous assays for phytoestrogens in biological fluids have analyzed a limited number of analytes and have often suffered from complex, multistage sample preparation techniques, questionable accuracy, poor precision, and insufficient sensitivity to analyze samples from subjects consuming a traditional Western diet. Using two recently developed, highly sensitive gas chromatography/mass spectrometry (MS) and liquid chromatography/MS methods, it is now possible to measure urinary and circulating levels of phytoestrogens more accurately. Accuracy has been improved by the use of triply ^{13}C-labeled standards (49, 50), superior sensitivity, and a simplified sample preparation procedure, which leads to less sample to sample variation.

In view of the difficulties encountered with obtaining accurate food composition data to calculate intakes of phytoestrogens from food consumption data, we have used these improved assays for seven of the phytoestrogens to examine levels in serum and urine as replacement biomarkers of intake. We have then examined the relationship between levels of each of these compounds and risk of breast cancer in women taking part in the Norfolk cohort of the European Prospective Investigation of Cancer and Nutrition (EPIC). Information on dietary intake of genistein and daidzein was also collected and related to breast cancer risk and the isoflavone replacement biomarkers.

Subjects and Methods

Study Population. In EPIC-Norfolk, 16,744 women aged 41–76 years, resident in Norfolk, United Kingdom, were recruited in 1993–1997 using general practice age sex registers. Permission for the study was obtained from the Norfolk and Norwich Hospital Ethics Committee. Of these, 13,070 women attended a medical examination and gave blood and an untimed spot urine sample at a clinic (51). The urine samples were stored at −20°C until analyzed for creatinine and phytoestrogens. The serum samples were stored at −40°C until analyzed for phytoestrogens. From 1993 to July 2001, 172 incident cases of breast cancer in women were recorded in EPIC participants by the East Anglian Cancer Registry for whom two controls were selected for each case, matched on age and date of recruitment. One hundred fourteen of the cases had attended the clinic, completed a 7-day diary of all food and drink consumed (52, 53), and given a spot urine sample.

Dietary Data. Information from the 7-day dietary diaries was used to calculate dietary fiber intake as nonstarch polysaccharides (53) using a custom-designed dietary assessment software program, Data into Nutrients for Epidemiological Research (54). Lignans occur ubiquitously in plant foods as minor constituents and would be expected to correlate with fiber content of plant foods. Hence, fiber intake was calculated as a surrogate indicator for lignan intake, as information on dietary lignan intake was not available. Dietary isoflavone intakes were determined using a food composition database based on daidzein and genistein concentrations

measured in 300 commonly eaten foods. Details on the sampling of foods and analysis of daidzein and genistein and their contents in different foods have been reported elsewhere (55–58). Isoflavone content of foods gathered from a literature search of published values was also incorporated into the food composition database for use in the analysis. The food composition database of isoflavones used in this study represents United Kingdom's contribution to the Vegetal Estrogens in Nutrition and the Skeleton database, a regional food composition database established to facilitate the estimation of exposure levels to phytoestrogens in four European countries, including Italy, the Netherlands, Ireland, and the United Kingdom (59, 60). The first day of the dietary information covered the day before the urine and blood samples were collected at the clinic.

Urinary Analysis. Of the two healthy controls selected for the 114 cases, 219 had donated a spot urine sample, which was included in the analyses. The remaining nine controls (selected as above) did not supply a urine sample. Urinary creatinine concentrations were measured based on a kinetic modification of the Jaffe reaction using the Roche reagent for creatinine on a Roche Cobas Mira Plus chemistry analyzer (Roche Products Ltd., Hertfordshire, United Kingdom).

The available spot urine samples (n = 333) were analyzed for three isoflavones (daidzein, genistein, and glycitein), two metabolites of daidzein (O-DMA and equol), and two lignans (enterodiol and enterolactone), blinded for case-control status. $^{13}C_3$-labeled standards in methanol were added to 200 μl sample, and conjugates were hydrolyzed to the aglycones, extracted on Strata C18-E SPE cartridges (Phenomenex, Macclesfield, United Kingdom), and derivatized to trimethylsilyl derivatives for analysis using isotope dilution gas chromatography/MS. Details and information on quality assurance and methodology have been reported elsewhere (49). Limits of detection range from 1.2 ng/ml (enterodiol) to 5.3 ng/ml (enterolactone). Non-equol producers were defined as those with values below the limit of detection of 1.3 ng/ml, corrected for dilution of sample (1.9 ng/ml).

Serum Phytoestrogen Analysis. Serum from blood collected at recruitment was available for 97 of 114 cases and for 187 controls. There was no spare serum available for the remaining 17 cases and 41 controls. These available serum samples (n = 284) were analyzed blinded as to case-control status for three isoflavones (daidzein, genistein, and glycitein), two metabolites of daidzein (O-DMA and equol), and two lignans (enterodiol and enterolactone). $^{13}C_3$-labeled standards in methanol were added to 200 μl sample, and conjugates were hydrolyzed to the aglycones, extracted on Strata C18-E SPE cartridges, dried under nitrogen, and redissolved in 40% methanol for analysis using isotope dilution liquid chromatography/tandem MS. Details and information on quality assurance and methodology have been reported elsewhere (50). Statistically calculated limits of detection range from 82 pg/ml (daidzein) to 222 pg/ml (equol). Non-equol producers were defined as those with serum equol levels higher than the limit of detection of 0.22 ng/ml, corrected for concentration of sample (0.11 ng/ml). Serum hormones were available for a subset of 219 women using methods described elsewhere (12).

Data Analysis. The basic statistical analyses were performed using SPSS software version 11.0 (SPSS UK Ltd., Surrey, United Kingdom). Urinary excretion was expressed as ng/ml of phytoestrogens and as μg/mmol of urinary creatinine. All dietary, urinary, and serum phytoestrogen data were skewed, so data were log transformed to create continuous variables (see example in Fig. 1) for all statistical tests. Pearson product moment correlations were used to assess the degree of association between urinary, serum, and dietary phytoestrogens. To assess whether urinary and serum phytoestrogens can serve as a valid dietary biomarker in epidemiological studies, we need to demonstrate differences in the distribution (*i.e.*, shifts in distribution) of the biomarker between subgroups of individuals with varying dietary intakes. Therefore, subjects were categorized into three groups according to tertiles of dietary intake of the respective phytoestrogen. Analyses were run using categorical variables scored from 1 to 3 according to

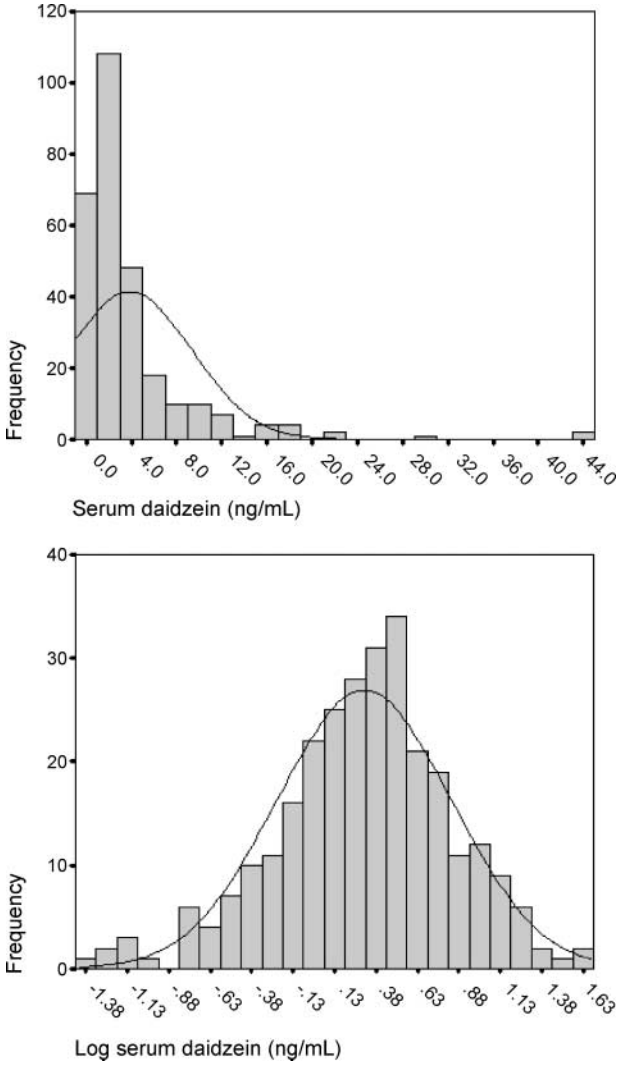

Figure 1. Histograms showing the frequency distributions of serum daidzein concentrations among study subjects (n = 284) before and after log transformation. Data were highly skewed and were corrected to a large extent by log transformation for the purpose of statistical testing.

Table 1. Dietary intake of fiber and dietary intake, urinary excretion (adjusted for creatinine concentration), and serum levels of phytoestrogens

	Geometric mean ± SD	Range*,†	Percentile†		
			25th	50th	75th
Dietary intake (μg/day), n = 331					
Isoflavones					
Daidzein	193.8 ± 2.4	0.4–6312.2	131.2	205.5	290.7
Genistein	243.4 ± 2.2	3.5–7675.4	167.4	247.2	350.8
Fiber (g/day)	13.3 ± 1.4	3.3–43.8	10.6	13.7	16.9
Urinary excretion (μg/mmol creatinine), n = 333					
Isoflavones					
Daidzein	16.0 ± 3.3	ND to 833.0	7.7	17.2	37.3
Genistein	8.6 ± 2.9	ND to 489.9	3.8	7.9	19.3
Glycitein	2.9 ± 2.4	ND to 56.0	0.8	2.5	6.3
O-DMA	0.8 ± 2.3	ND to 226.3	0.0	0.2	1.3
Equol	0.5 ± 2.1	ND to 119.7	0.0	0.1	0.7
Lignans					
Enterodiol	7.8 ± 2.6	ND to 438.5	3.5	7.8	16.1
Enterolactone	78.5 ± 3.7	ND to 9790.9	40.4	93.5	193.3
Serum levels (ng/ml), n = 284					
Isoflavones					
Daidzein	2.0 ± 3.4	0.1–44.8	1.0	2.2	4.5
Genistein	4.1 ± 2.7	ND to 159.7	1.3	3.5	8.5
Glycitein	0.1 ± 1.2	ND to 1.2	0.0	0.1	0.2
O-DMA	0.1 ± 1.5	ND to 31.9	0.0	0.0	0.2
Equol	0.2 ± 1.3	ND to 8.0	0.1	0.1	0.2
Lignans					
Enterodiol	0.4 ± 1.4	ND to 8.1	0.1	0.3	0.6
Enterolactone	3.8 ± 3.6	ND to 388.1	2.3	4.5	8.7

*ND indicates that the analyte was not detected (*i.e.*, zero value or value below the limit of detection of the assay). Limits of detection (ng/ml) for urine analyses by GC/MS after correction for dilution of sample were as follows: daidzein 1.9, genistein 1.9, glycitein 6.5, O-DMA 7.5, equol 1.9, enterodiol 1.8, and enterolactone 8.0. Limits of detection (ng/ml) for serum analyses by LC/MS after correction for concentration of sample were as follows: daidzein 0.04, genistein 0.06, glycitein 0.06, O-DMA 0.06, equol 0.11, enterodiol 0.08, and enterolactone 0.05.
†Based on untransformed data.

which intertertile interval an observation lay. ANOVA was used to compare differences in urinary and serum phytoestrogen concentrations among subjects in different tertiles of dietary intake. Trend tests were computed using these tertile-based scores to assess the dose-response relationship between dietary intake, serum concentrations, and urinary excretion. All P values are two sided and $P < 0.05$ was considered statistically significant. Results are reported as cases and controls combined because results were similar to analyses on cases and controls analyzed separately.

For the calculation of odds ratio for breast cancer risk, the statistical analyses were performed using conditional logistic regression (Stata version 7.0, Stata Corp., TX) for the urine and plasma separately. All data for all phytoestrogens were transformed to $\log_2$ so that the risk estimates would represent a doubling in phytoestrogen exposure (12). Models were adjusted for weight, height, parity, menopausal status, smoking history, family history of breast cancer, hormone replacement therapy use, and saturated fat consumption (58).

Results

Mean (SD) age, body mass index, and age at birth of first child were identical in cases and controls [age: controls 58.8 (8.4), cases 58.7 (8.5) years; body mass index: controls 26.5 (4.0), cases 26.7 (4.6) kg/m^2; age at birth of first child: controls 25.0 (4.4), cases 25.0 (4.0) years]. The majority of women were postmenopausal (60%), never smokers (58%), never hormone replacement therapy users (66%), and with no family history of breast cancer (81%). There were no significant differences in phytoestrogen levels between women of different menopausal status. Mean (SD) plasma levels of estradiol were 24.9 (15.9) pmol/l (0.006 ng/ml) in the 135 women defined as postmenopausal by both hormone profile (plasma follicle stimulation hormone concentration > 30 IU/l and plasma estradiol concentration < 100 pmol/l) and self-report of no menstrual period for at least 5 years.

Table 1 shows that the dietary isoflavone intake of subjects was low, with an average daily intake of 437 μg and a median intake of 423 μg. Even those subjects at the 95th percentile consumed only 1.2 mg/day (data not shown). Only 3% of the population consumed soy foods and 61% of isoflavones were derived from bread and bakery products. The isoflavone intake composed of daidzein and genistein in almost equal amounts. Intake of non-starch polysaccharides was similar to the EPIC average (SD) of 13.3 (1.4) g for this age group of women (53).

Daidzein was the predominant isoflavone excreted in urine, with urinary daidzein concentrations being twice as high as that of genistein. Median values (ng/ml) uncorrected for creatinine were O-DMA 0.96, daidzein 88.1, genistein 38.9, glycitein 11.4, equol 0.26, enterodiol 34.4, and enterolactone 462. Using the limit of detection of equol in urinary analysis as a cutoff value, 114 of 333 (34%) subjects had urinary equol higher than the limit of detection of 1.3 ng/ml, corrected for dilution of sample (1.9 ng/ml).

Table 2. Correlation matrix of urinary phytoestrogen excretion and serum phytoestrogen levels (n = 284–333)

	Urinary excretion without (a) and with (b) adjustment for urinary creatinine concentration (ng/ml; μg/mmol creatinine)							
	Daidzein		Genistein		Glycitein		O-DMA	
	a	b	a	b	a	b	a	b
Serum levels (ng/ml)								
Isoflavones	0.72*	0.84*	0.75*	0.88*	0.63*	0.76*	0.43*	0.47*
Daidzein	**0.80***	**0.91***	0.69*	0.79*	0.64*	0.77*	0.46*	0.46*
Genistein	0.66*	0.76*	**0.76***	**0.89***	0.60*	0.71*	0.35*	0.39*
Glycitein	0.61*	0.68*	0.62*	0.68*	**0.68***	**0.81***	0.36*	0.33*
O-DMA	0.36*	0.47*	0.26*	0.34*	0.27*	0.36*	**0.66***	**0.82***
Equol	0.11	0.18‡	0.16‡	0.24*	0.08	0.15†	0.18‡	0.22*
Lignans	−0.02	0.03	−0.13†	−0.10	−0.11	−0.09	0.04	0.05
Enterodiol	0.05	0.08	0.00	0.03	0.04	0.08	−0.01	0.01
Enterolactone	−0.03	−0.01	−0.15†	−0.13†	−0.13†	−0.13†	0.02	0.01

Note: Pearson correlation coefficients on log-transformed data. Correlation coefficients between urine and serum levels are indicated in bold for particular phytoestrogens.
*$P < 0.001$.
†$P < 0.05$.
‡$P < 0.01$.

The ratio of genistein to daidzein was reversed when urine was compared with blood. In serum, concentrations of genistein were more than twice as high as that of serum daidzein, in contrast to the urine ratios. The mean value of serum genistein was 4.1 ng/ml (15.2 nmol/l), which was ~600 times the mean levels of estradiol in postmenopausal women. Enterolactone was the predominant lignan, with concentrations more than 10 times higher than that of enterodiol in both urine and serum. There were also differences between urine and blood in the ratio of isoflavones to lignans. In urine, excretion of lignans was greater, reflecting the larger amounts of lignans consumed in Western populations (1). In urine, the concentration of lignans was more than three times higher than that of isoflavones, while in serum, lignan concentration was only about half of the isoflavone concentration (Table 1). One hundred seven of 284 (38%) subjects had serum equol levels higher than the limit of detection of 0.22 ng/ml, corrected for concentration of sample (0.11 ng/ml).

Table 2 shows that the spot urine concentrations of all phytoestrogens (adjusted for urinary creatinine concentration) correlated strongly with serum concentrations. Correlation coefficients ranged from 0.81 between serum and urine glycitein and equol to as high as 0.94 between serum and urine enterolactone. Scatter plots showed clear linear relationships between concentrations of phytoestrogens in spot urine and serum (see example in Fig. 2). Correlation coefficients between urine and serum concentrations were consistently higher when urine concentrations were adjusted for creatinine concentration compared with the unadjusted values. The isoflavones generally showed significant intercorrelation (*e.g.* serum glycitein was significantly correlated with urinary daidzein, genistein, glycitein, and O-DMA). The two lignans, enterodiol and enterolactone, were also significantly correlated with each other. However, equol was poorly correlated with all other phytoestrogens (Table 2). Correlation coefficients between equol and daidzein were 0.21 ($P < 0.001$) and 0.20 ($P = 0.001$) in urine and serum, respectively (data not shown). Figure 3 shows that although serum and urine equol levels were highly correlated, the agreement was less good at low levels. Hence, 180 of 284 (63%) subjects had detectable equol in either urine or serum, but only 55 (19%) subjects had detectable equol in both urine and serum.

Table 3 shows that urine and serum concentrations of daidzein, genistein, and total isoflavones correlated significantly ($P < 0.001$) with their dietary intakes as assessed from the 7-day food diaries. Correlation between the metabolites equol and O-DMA and their dietary precursor daidzein was poor, with urinary equol not showing a significant correlation and serum equol only a weak correlation with dietary daidzein. No data on dietary intake of lignans was available, but urinary and serum lignan concentrations correlated significantly ($P < 0.01$) with dietary fiber intake, with correlation

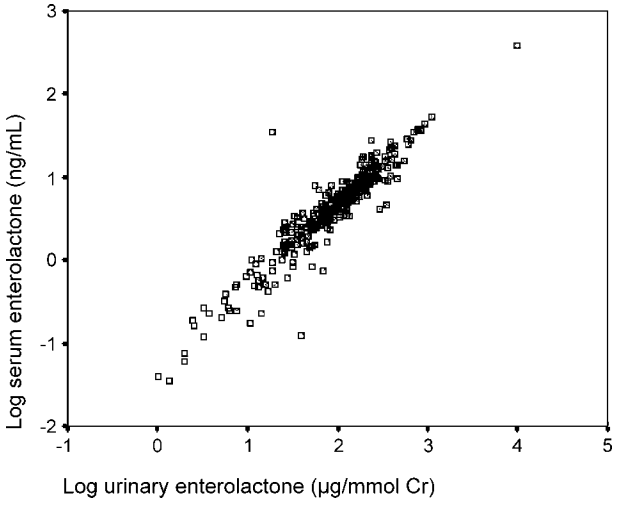

Figure 2. Scatter plot of concentration of enterolactone in spot urine sample (adjusted for urinary creatinine concentration) *versus* serum sample (n = 284). Pearson correlation = 0.94 ($P < 0.001$).

Table 2. Correlation matrix of urinary phytoestrogen excretion and serum phytoestrogen levels (n = 284–333) (Cont'd)

Urinary excretion without (a) and with (b) adjustment for urinary creatinine concentration (ng/ml;μg/mmol creatinine)

Equol		Isoflavones		Enterodiol		Enterolactone		Lignans	
a	b	a	b	a	b	a	b	a	b
0.14†	0.19‡	**0.74***	**0.85***	0.07	0.02	−0.03	−0.07	0.01	−0.04
0.11	0.15†	0.76*	0.89*	0.05	0.03	0.01	−0.01	0.03	0.00
0.13†	0.18‡	0.70*	0.79*	0.06	0.01	−0.04	−0.09	0.00	−0.06
0.10	0.11	0.61*	0.66*	0.12†	0.05	−0.07	−0.16‡	−0.02	−0.12†
0.04	0.06	0.36*	0.45*	−0.02	−0.01	−0.02	−0.01	0.00	0.02
0.62*	0.81*	0.17‡	0.24*	−0.04	−0.01	0.02	0.06	0.01	0.06
0.03	0.05	−0.06	−0.02	0.37*	0.47*	0.77*	0.91*	**0.76***	**0.93***
0.02	0.04	0.04	0.07	**0.67***	**0.83***	0.23*	0.28*	0.33*	0.40*
0.02	0.03	−0.08	−0.06	0.27*	0.34*	**0.81***	**0.94***	0.75*	0.90*

coefficients ranging from 0.17 (urine) to 0.23 (serum). There was no correlation between equol concentration and fiber intake. The correlation between urine and dietary values was somewhat higher for the first day of diary information (r = 0.32 for daidzein, 0.34 for genistein, and 0.26 for enterolactone and fiber), but this effect was not consistent for serum levels or isoflavone metabolites (data not shown).

Table 4 shows that when women were divided into tertiles according to their intakes daidzein and genistein, respectively, there were significant differences ($P < 0.05$) in urinary and serum concentrations of these compounds between women in the highest tertiles and the lowest tertiles (except for serum daidzein where P = 0.054). Trend tests showed significant dose-response relationships ($P < 0.02$) between both urinary and serum concentrations of the isoflavones across increasing tertiles of dietary intakes. Figure 4 shows the strong relationship between dietary daidzein intake and log urinary daidzein. A similar pattern was found between urinary and serum lignan concentrations in women grouped according to tertiles of fiber intake (Table 4).

Table 5 shows odds ratios of breast cancer risk according to phytoestrogens in serum and urine with data transformed to $\log_2$. All the isoflavones were associated with increased breast cancer risk and the odds ratio for serum daidzein was significant (P = 0.044). Mean unadjusted daidzein in serum was 4.65 ng/ml for cases and 3.50 ng/ml for controls (median = 2.42 and 2.11 ng/ml, respectively). Thirty-three cases of 95 women (35%) were classified in the top tertile for daidzein serum levels compared with 27 cases of 95 women (28%) in the bottom tertile. The unadjusted odds ratio for daidzein in serum was 1.173 (0.995–1.383; P = 0.057).

The odds ratios for equol in both urine and serum were also significant (P = 0.013 and 0.024, respectively). The unadjusted odds ratio was 1.239 (0.995–1.542; P = 0.055) for equol in urine and was 1.343 (1.012–1.783; P = 0.041) for equol in serum. Mean unadjusted equol excretion in urine was 2.54 μg/mmol creatinine for cases and 1.30 μg/mmol creatinine for controls (median = 0.15 and 0.07 μg/mmol creatinine, respectively). Due to the inequalities in numbers of producers *versus* non-equol producers, it was not possible to classify individuals into tertiles of excretion, but for urine, 45 cases of 114 women (39%) were equol producers compared with 69 cases of 219 women (31%) who were nonproducers. Mean unadjusted equol in serum was 0.29 ng/ml for cases and 0.18 ng/ml for controls (median = 0.10 and 0.09 ng/ml, respectively). For serum, 38 cases of 97 women (39%) were equol producers compared with 69 cases of 187 women (37%) who were nonproducers.

Odds ratios for phytoestrogen excretion in urine without correction for creatinine excretion (as ng/ml) were similar (data not shown). Adding estradiol as a covariate on a subset (n = 219 urine and 189 plasma) of the study for which it was available reduced significance due to a smaller number of samples and showed some

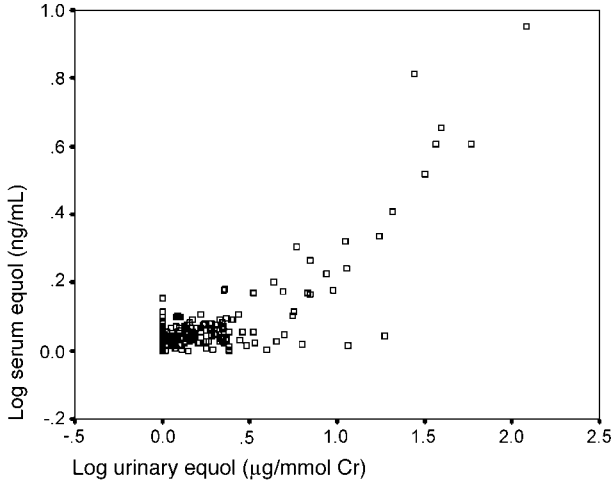

Figure 3. Scatter plot of concentration of equol in spot urine sample (adjusted for urinary creatinine concentration) *versus* serum sample (n = 284).

Table 3. Correlation matrix of dietary isoflavones and fiber (non-starch polysaccharides) intake from 7-day food diaries and urinary and serum phytoestrogen levels (n = 284–333)

		Dietary intake from 7-day food diaries			
		Daidzein (μg/day)	Genistein (μg/day)	Isoflavones (μg/day)	Dietary fiber non-starch polysaccharides (g/d)
Urinary levels (μg/mmol creatinine)	Isoflavones	0.26*	0.26*	**0.27***	0.00
	Daidzein	**0.26***	0.27*	0.27*	0.03
	Genistein	0.29*	**0.29***	0.30*	−0.01
	Glycitein	0.21*	0.21*	0.21*	−0.06
	O-DMA	0.17†	0.19*	0.20*	−0.04
	Equol	0.09	0.10	0.10	−0.04
	Lignans	0.05	0.06	0.06	**0.17†**
	Enterodiol	0.14‡	0.14‡	0.13‡	0.13‡
	Enterolactone	0.06	0.07	0.07	0.29*
Serum levels (ng/ml)	Isoflavones	0.29*	0.31*	**0.31***	0.05
	Daidzein	**0.25***	0.27*	0.27*	0.07
	Genistein	0.29*	**0.32***	0.32*	0.05
	Glycitein	0.12‡	0.12‡	0.12‡	−0.07
	O-DMA	0.21*	0.23*	0.24*	0.00
	Equol	0.14‡	0.15‡	0.15†	0.04
	Lignans	0.05	0.06	0.06	**0.23***
	Enterodiol	0.07	0.07	0.07	0.03
	Enterolactone	0.03	0.04	0.04	0.22*

Note: Pearson correlation coefficients on log-transformed data. Correlation coefficients between dietary and urine and serum levels are indicated in bold for particular phytoestrogens. Dietary fiber is used as a surrogate indicator of dietary lignan intake.
*$P < 0.001$.
† $P < 0.01$.
‡ $P < 0.05$.

attenuation for equol but none for daidzein (data not shown). The $\log_2$ odds ratios for breast cancer risk according to dietary intake of daidzein and genistein were also elevated but not significantly so [1.176 (0.933–1.482; P = 0.352) for daidzein adjusted, 1.195 (0.975–1.464; P = 0.086) daidzein unadjusted; 1.165 (0.938–1.447; P = 0.166) genistein adjusted; 1.188 (0.978–1.444; P = 0.081) genistein unadjusted].

Discussion

The epidemiological assessment of the health effects of phytoestrogens in populations consuming low levels has been made difficult in the past due to insufficient sensitivity in analytical methods used to assess very low levels in biological samples. With the advent of the highly sensitive and accurate MS techniques used here, incorporating triply ^{13}C-labeled standards (49, 50), we have been able to assess seven phytoestrogens simultaneously in blood and urine samples collected from one of the largest prospective studies thus far conducted of phytoestrogens and breast cancer in the EPIC-Norfolk cohort (52). In addition, using our previous analyses of levels of isoflavones in foods (55–58), we have been able to compose a database and obtain estimates of dietary intake from detailed prospective diary records of food intake (53, 54).

The dietary isoflavone intakes of women in this study were found to be low, with a mean intake of only 437 μg/day. This isoflavone intake is comparable with that reported for American women (0.63 mg/day; Ref. 61) but considerably lower than that reported for Singapore women (4.7 mg/day; Ref. 37), Chinese women (33.4 mg/day; Ref. 38), and Japanese women (46.5 mg/day; Ref. 39) and reflects the low habitual soy consumption among EPIC-Norfolk women and Western populations in general. In EPIC-Norfolk, only 3% of the population consumed soy foods and 61% of isoflavones were derived from bread and bakery products, where soy products are used as emulsifiers and to extend shelf life. Urinary isoflavone excretion was low (28.8 μg/mmol creatinine or 1.0 nmol/mg creatinine) and is comparable with that reported for American women (0.7 nmol/mg creatinine; Ref. 46). Serum isoflavone concentrations were also low. The combined serum daidzein and genistein concentration was 6.5 ng/ml (23.0 nmol/l)

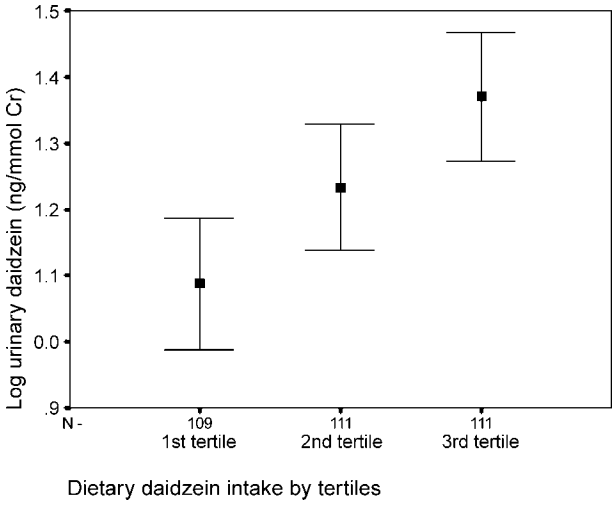

Figure 4. Error bar plot (means and 95% confidence intervals) of urinary daidzein concentrations of women across each tertile of dietary daidzein intake.

Table 4. Urine and serum levels of phytoestrogens among women grouped according to tertiles of dietary intakes (*n* = 284–333)

Tertiles of dietary intake	Urinary levels (μg/mmol creatinine)			Serum levels (ng/ml)		
	Geometric mean (SD)	*P* for ANOVA	*P* for trend	Geometric mean (SD)	*P* for ANOVA	*P* for trend
Daidzein	Daidzein	<0.001	<0.001	Daidzein	0.054	0.016
1st tertile	11.5 (3.4)			1.6 (3.3)		
2nd tertile	16.1 (3.2)			2.0 (3.4)		
3rd tertile	22.4 (3.3)			2.5 (3.4)		
Genistein	Genistein	<0.001	<0.001	Genistein	0.001	<0.001
1st tertile	6.5 (2.8)			5.0 (2.3)		
2nd tertile	7.9 (2.8)			5.5 (2.2)		
3rd tertile	12.8 (2.9)			7.7 (2.4)		
Fiber	Lignans	<0.001	<0.001	Lignans	0.003	0.001
1st tertile	66.9 (2.8)			3.5 (2.7)		
2nd tertile	87.0 (3.3)			4.2 (3.2)		
3rd tertile	140.2 (3.3)			6.0 (3.1)		

and is comparable with that reported for American women (19.1 nmol/l; Ref. 43). Despite the low level of intake, the mean serum level of genistein was up to 600 times greater than postmenopausal estradiol levels, approaching estrogenic levels found in *in vivo* assays (1).

In this study, we found that isoflavone concentrations in spot urine and serum were significantly correlated to dietary intake. Correlation coefficients between dietary isoflavone intake and their concentrations in spot urine and serum dietary intakes ranged from 0.25 to 0.32 (Table 3). Urinary recovery of daidzein and genistein from a single time point intake is reported to be complete within 24–36 h (62) and serum elimination half-lives of daidzein and genistein are reported to be 8–10 h (63). Studies elsewhere comparing intake with urine or serum isoflavone concentrations (36, 38–43, 45–47) have reported correlation coefficients ranging from −0.05 (P = 0.85) for urinary daidzein *versus* dietary daidzein in 18 Caucasian women in Los Angeles with isoflavone intake of 2.2 mg/day (45) to 0.62 (P = 0.0001) for urinary isoflavones *versus* dietary isoflavones in 102 multiethnic women in Hawaii with isoflavone intakes of 5.0–38.2 mg/day (41). In one study with subjects selected based on prior knowledge of soy intake to capture a wide variation in soy consumption, the Spearman correlation coefficient was as high as 0.80 (44). In general, the correlation between intake and urine or serum isoflavones appears to be higher in populations with higher isoflavone intakes and greater variation in intakes.

The relatively poor correlation between dietary and urinary and serum isoflavones found in this study and elsewhere in low soy-consuming populations is likely due to measurement error in the dietary data. Prediction of accurate intakes from databases of food composition is very difficult due to variability in levels in foods and limited analytical data (31). In addition, soy additives added to processed foods can contribute substantially to its isoflavone content so that a wide variety of processed foods contain appreciable amounts of isoflavones (64). It is hard for food composition databases to keep up with the proliferation of soy additives in food and prepre-pared foods, functional foods, and dietary supplements. Correlation coefficients are also bound to be lower in studies where the extent of individual variation is low than in studies where it is high. However, we did observe statistically significant dose-response shifts in distributions of urinary and serum isoflavones between women in increasing tertiles of dietary intakes despite the low isoflavone intake in our study population (Table 4). Data on lignan intake were not available, but Table 3 shows that the dietary fiber intake and lignan concentrations in spot urine and serum samples correlated in a similar manner and to a similar degree to that observed between dietary isoflavones and isoflavone

Table 5. Log_2 odds ratio (associated with a doubling of exposure) for urine and serum phytoestrogen levels: conditional logistic regression adjusting for body mass index, menopausal status, parity, hormone replacement therapy use, smoking, family history of breast cancer, and saturated fat intake

Phytoestrogen	Log_2 odds ratio for urine and serum phytoestrogen levels	
	Urine (adjusted for creatinine output)	Serum
O-DMA	1.148 (0.930–1.417; P = 0.198)	1.140 (0.933–1.393; P = 0.199)
Daidzein	1.123 (0.963–1.309; P = 0.138)	1.220 (1.005–1.481; P = 0.044)
Genistein	1.162 (0.973–1.387; P = 0.097)	1.237 (0.976–1.569; P = 0.077)
Glycitein	1.076 (0.869–1.333; P = 0.499)	1.226 (0.946–1.588; P = 0.123)
Equol	1.344 (1.063–1.699; P = 0.013)	1.455 (1.051–2.017; P = 0.024)
Enterodiol	1.015 (0.840–1.227; P = 0.873)	0.912 (0.738–1.126; P = 0.392)
Enterolactone	0.980 (0.850–1.130; P = 0.785)	0.996 (0.824–1.202; P = 0.963)

Note: The numbers of matched sets were as follows: for urine: total observations = 325 after the model has excluded missing values, 111 cases remain, 106 of which have two matched controls and 5 of which have 1 matched control; for serum, total observations = 255 after the model has excluded missing values, 92 cases remain, 89 of which have two controls and 3 of which have 1 control.

concentrations in spot urine and serum. These findings are similar to those found elsewhere and suggest that fiber may be suitable as a surrogate indicator of lignan intake (47, 65). It also implies that the protective effect of fiber on disease risk found in some studies may be in part due to lignans or vice versa. Although it has been suggested that equol production may be stimulated through increased microbial flora activity in the large gut when fiber intakes are increased (48), we were not able to show evidence of a relation between fiber intake and equol production (Table 3).

In this study, we were surprised by the strong correlations between phytoestrogen levels in the spot urine and those found in serum, with correlation coefficients ranging from 0.81 for equol and glycitein to 0.94 for enterolactone (Table 3). This was true even of genistein and daidzein, the rankings in concentration of which were reversed when serum was compared with urine excretion, and of the lignan/isoflavone ratio, which was reversed in plasma when compared with urine (Table 1). There are little data available on the pharmacokinetics of phytoestrogens to explain these reversals (1). Few studies elsewhere have investigated the relationship between phytoestrogen concentrations in urine and serum. Of the two studies reported, both studies were carried out in Japanese subjects using 24 h urine collections. They reported correlation coefficients ranging from 0.23 to 0.45 for daidzein and from 0.34 to 0.50 for genistein between urine and serum (39, 40). The much higher correlations reported here, although only a spot urine was available, and the fact that little soy was consumed may reflect the accuracy and sensitivity of our analytical techniques. A potential problem associated with the use of spot urine samples is that the concentration of the analyte of interest is dependent on urine dilution. To adjust for variability in urine dilution, a commonly used method (37, 38, 46) is to normalize analyte concentrations to urinary creatinine, which is a compound that is excreted by glomerular filtration at a relatively constant rate. In this study, correcting for creatinine excretion improved associations, as would be expected.

Although equol and O-DMA in serum and urine are metabolites of dietary daidzein, they showed poor correlation with daidzein intake. No significant correlation was found between urinary equol and dietary daidzein. This is likely because gut microflora plays an important role in determining equol levels in the body (1). Several studies have suggested that only approximately one-third of the population are capable of equol production (1). In this study, we used the limits of detection of our assays as cutoff values and estimated that a similar proportion, 34–38% of our study population, may be equol producers based on separate analyses of spot urine and serum. However, there is no clear consensus on the definition of equol producers. In soy challenge studies, different cutoff values for equol excretion have been used in defining equol producers (48, 66, 67). At low levels, serum and urine values are less well correlated, and between 19% and 63% subjects could be classified as equol producers based on whether they had values in only one, or one of either, sample. No other epidemiological studies carried out on Western populations have assessed equol production due to lack of an immunoassay technique at that time and poor ionization of equol by liquid chromatography/MS (68).

Studies elsewhere of phytoestrogen intakes in relation to breast cancer risk have been mostly case-control studies (13–18), many of which were done in populations consuming high amounts of soy (13, 15–17). For the assessment of individual risk in analytic epidemiology, case-control studies are subject to certain biases, because measurements are made after disease occurrence and prospective studies of initially healthy subjects are preferred. The linear dose-response relationships between urine and serum levels of phytoestrogens across increasing tertiles of dietary phytoestrogen intake found here suggest that either can be used as a biomarker of intake in prospective investigations if dietary intake is not available (Table 4).

Breast cancer incidence rates are lower than in the Western in Far Eastern populations in which much soy are consumed (1–3). To explain this, it has been proposed that these compounds are antiestrogens and it would therefore be expected that a decreased risk of breast cancer would be associated with higher levels of isoflavones. However, contrary to expectations, the opposite was found in this study where smaller amounts of isoflavones, mainly as food additives especially in bakery products, were consumed. Levels of all the isoflavones were associated with an increase in risk of breast cancer, particularly daidzein and equol, which is produced from daidzein (1). The dietary daidzein and genistein intakes were also associated with increased risk, but not significantly so, probably because of the measurement error incurred with databases of food intakes referred to above. There were no effects of the lignans. Only one other prospective study has investigated biomarkers of phytoestrogen intakes in plasma or urine phytoestrogen levels (22). In this Netherlands prospective study of 88 cases and 268 controls, two phytoestrogens, genistein and enterolactone, in overnight urine samples were not significantly associated with differences in risk of breast cancer. Other phytoestrogens, including equol and daidzein, were not measured (22). No other case-control or prospective study of plasma or urine has measured all seven phytoestrogens simultaneously.

We have adjusted our estimates of risk from isoflavones for known confounding factors, and the increase in risk might have been predicted from the estrogenic effects of these compounds and their comparatively high levels in plasma when compared with endogenous sex hormones, which are known risk factors for breast cancer (1, 12) Nevertheless, our findings are based on relatively small numbers of cases and so could have arisen by chance. In this population, the consumption of soy containing foods, which would have increased the range of dietary intake levels substantially, was minimal. In addition, we cannot exclude residual confounding from other, unknown, factors. This could be examined in larger prospective studies in which information on known breast cancer risk factors and samples of blood or urine have been collected.

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References

1. Committee on Toxicity of Chemicals in Food/Consumer Products and the Environment. COT Report—Phytoestrogens and Health. London: Food Standards Agency; 2003.

2. Setchell KDR, Borriello SP, Hulme P, Kirk DN, Axelson M. Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. Am J Clin Nutr 1984;40:569-78.
3. Adlercreutz, H. Does fiber-rich food containing animal lignan precursors protect against both colon and breast cancer? An extension of the ''fiber hypothesis.'' Gastroenterology 1984;86:761-4.
4. Kuiper G, Lemmen J, Carlsson B, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 1998;139:4252-63.
5. Tang B, Adams N. Effects of equol on estrogen receptors and on synthesis of DNA and protein in the immature rat uterus. J Endocrinol 1980;85:291-7.
6. Waters A, Knowler J. Effect of a lignan (HPMF) on RNA synthesis in the rat uterus. J Reprod Fertil 1982;66:379-81.
7. Wei H, Bowen R, Cai Q, Barnes S, Wang Y. Anti-oxidant and antipromotional effects of the soybean isoflavone genistein. Proc Soc Exp Biol Med 1995;208:124-30.
8. Prasad K. Hydroxyl radical-scavenging property of secoisolariciresinol diglucoside (SDG) isolated from flax-seed. Mol Cell Biochem 1997;168:117-23.
9. Akiyama T, Ishida J, Nakagawa S, et al. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 1987;262:5592-5.
10. Markovits J, Linassier C, Fosse P, et al. Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. Cancer Res 1989;49:5111-7.
11. Kirk CJ, Harris RM, Wood DM, Waring RH, Hughes PJ. Do dietary phytoestrogens influence susceptibility to hormone-dependent cancer by disrupting the metabolism of endogenous estrogens? Biochem Soc Trans 2001;29:209-16.
12. Endogenous Hormones and Breast Cancer Collaborative Group. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 2002;94:606-16.
13. Lee H, Gourley L, Duffy S, Esteve J, Day NE. Dietary effects on breast-cancer risk in Singapore. Lancet 1991;337:1197-200.
14. Ingram D, Sanders K, Kolybaba M, Lopez D. Case-control study of phyto-estrogens and breast cancer. Lancet 1997;350:990-4.
15. Wu A, Ziegler R, Horn-Ross L, et al. Tofu and risk of breast cancer in Asian-Americans. Cancer Epidemiol Biomarkers & Prev 1996;5:901-6.
16. Hirose T, Tajima K, Hamajima N, et al. A large scale, hospital based case-control study of risk factors of breast cancer according to menopause status. Jpn J Cancer Res 1995;86:146-54.
17. Shu X-O, Jin F, Dai Q, et al. Soyfood intake during adolescence and subsequent risk of breast cancer among Chinese women. Cancer Epidemiol Biomarkers & Prev 2001;10:483-8.
18. Murkies A, Dalais FS, Briganti E, et al. Phytoestrogens and breast cancer in postmenopausal women: a case control study. Menopause 2000;7:289-96.
19. Hargreaves DF, Potten CS, Harding C, et al. Two-week dietary soy supplementation has an estrogenic effect on normal pre-menopausal breast. J Clin Endocrinol Metab 1999;84:4017-24.
20. Nomura AMY, Henderson B, Lee J. Breast cancer and diet among the Japanese in Hawaii. Am J Clin Nutr 1978;31:2020-5.
21. Greenstein J, Kushi L, Zheng W, et al. Risk of breast cancer associated with intake of specific foods and food groups. Am J Epidemiol 1996;145:S36.
22. den Tonkelaar I, Keinan-Boker L, Van't Veer P, et al. Urinary phytoestrogens and postmenopausal breast cancer risk. Cancer Epidemiol Biomarkers & Prev 2001;10:223-8.
23. Horn-Ross P, Hoggatt KJ, West DW, et al. Recent diet and breast cancer risk: the California Teachers Study (USA). Cancer Causes & Control 2002;13:407-15.
24. Key T, Sharp G, Appleby P, et al. Soya foods and breast cancer risk: a prospective study in Hiroshima and Nagasaki, Japan. Br J Cancer 1999;81:1248-56.
25. Hirayama T. Lifestyle and mortality: a large scale census-based cohort study in Japan. Contributions to epidemiology and biostatistics. Basel: Karger; 1990.
26. Yamamoto S, Sobue T, Kobayashi M, Sasaki S, Tsugane S. Soy, isoflavones and breast cancer risk in Japan. J Natl Cancer Inst 2003;95:906-13.
27. Eldrige A, Kwolek W. Soybean isoflavones: effect of environment and variety on composition. J Agric Food Chem 1983;31:394-6.
28. Wang H-J, Murphy PA. Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year and location. J Agric Food Chem 1994;42:1674-7.
29. Wang H-J, Murphy PA. Isoflavone content in commercial soybean foods. J Agric Food Chem 1994;42:1666-73.
30. Thompson LU, Rickard SE, Cheung F, Kenaschuk EO, Obermeyer WR. Variability in anticancer lignan levels in flaxseed. Nutr Cancer 1997;27:26-30.
31. Setchell KD, Cole SJ. Variations in isoflavone levels in soy foods and soy protein isolates and issues related to isoflavone databases and food labeling. J Agric Food Chem 2003;51:4146-55.
32. Zheng W, Dai Q, Custer LJ, et al. Urinary excretion of isoflavonoids and the risk of breast cancer. Cancer Epidemiol Biomarkers & Prev 1999;8:35-40.
33. Pietinen P, Stumpf K, Männistö S, Kataja V, Uusitupa M, Adlercreutz H. Serum enterolactone and risk of breast cancer: a case-control study in Eastern Finland. Cancer Epidemiol Biomarkers & Prev 2001;10:339-44.
34. Dai Q, Franke AA, Jin F, et al. Urinary excretion of phytoestrogens and risk of breast cancer among Chinese women in Shanghai. Cancer Epidemiol Biomarkers & Prev 2002;11:815-21.
35. Karr S, Lampe JW, Hutchins A, Slavin J. Urinary isoflavonoid excretion in humans is dose-dependent at low to moderate levels of soy protein consumption. Am J Clin Nutr 1997;66:46-51.
36. Adlercreutz H, Honjo H, Higashi A, et al. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. Am J Clin Nutr 1991;54:1093-100.
37. Seow A, Shi CY, Franke AA, Hankin JH, Lee H-P, Yu MC. 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 Biomarkers & Prev 1998;7:135-40.
38. Chen Z, Zheng W, Custer LJ, et al. 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 1999;33:82-7.
39. Arai Y, Uehara M, Sato Y, et al. Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. J Epidemiol 2000;10:127-35.
40. Yamamoto S, Sobue T, Sasaki S, et al. Validity and reproducibility of a self-administered food-frequency questionnaire to assess isoflavone intake in a Japanese population in comparison with dietary records and blood and urine isoflavones. J Nutr 2001;131:2741-7.
41. Maskarinec G, Singh S, Meng L, Franke AA. Dietary soy intake and urinary isoflavonoid excretion among women from a multiethnic population. Cancer Epidemiol Biomarkers & Prev 1998;7:613-9.
42. Frankenfeld CL, Patterson RE, Kalhorn TF, Skor HE, Howald WN, Lampe JW. Validation of a soy food frequency questionnaire with plasma concentrations of isoflavones in U.S. adults. J Am Diet Assoc 2002;102:1407-13.
43. Frankenfeld CL, Patterson RE, Horner NK, et al. Validation of a soy food-frequency questionnaire and evaluation of correlates of plasma isoflavone concentrations in postmenopausal women. Am J Clin Nutr 2003;77:674-80.
44. Verkasalo P, Appleby P, Allen N, Davey G, Adlercreutz H, Key T. Soya intake and plasma concentrations of daidzein and genistein: validity of dietary assessment among eighty British women (Oxford Arm of the European Prospective Investigation into Cancer and Nutrition). Br J Nutr 2001;86:415-21.
45. Huang M-H, Harrison GG, Mohamed MM, et al. Assessing the accuracy of a food frequency questionnaire for estimating usual intake of phytoestrogen. Nutr Cancer 2000;37:145-54.
46. Atkinson C, Skor HE, Fitzgibbons ED, et al. Overnight urinary isoflavone excretion in a population of women living in the United States, and its relationship to isoflavone intake. Cancer Epidemiol Biomarkers & Prev 2002;11:253-60.
47. Lampe JW, Gustafson DR, Hutchins AM, et al. Urinary isoflavonoid and lignan excretion on a western diet: relation to soy, vegetable, and fruit intake. Cancer Epidemiol Biomarkers & Prev 1999;8:699-707.
48. Setchell KDR, Brown NM, Lydeking-Olsen E. The clinical importance of the metabolite equol-a clue to the effectiveness of soy and its isoflavones. J Nutr 2002;132:3577-84.
49. Grace PB, Taylor JI, Botting NP, Fryatt T, Oldfield MF, Bingham SA. Quantification of isoflavones and lignans in urine using gas chromatography/mass spectrometry. Anal Biochem 2003;315:114-21.
50. Grace PB, Taylor JI, Botting NP, et al. Quantification of isoflavones and lignans in serum using isotope dilution liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectr 2003;17:1350-7.
51. Riboli E. Nutr Cancer: background and rationale of the European Prospective Investigation into Cancer and Nutrition (EPIC). Ann Oncol 1992;3:783-91.
52. Day NE, Oakes S, Luben R, et al. EPIC-Norfolk: study design and characteristics of the cohort. Br J Cancer 1999;80(Suppl 1):95-103.

53. Bingham SA, Welch AA, McTaggart A, et al. Nutritional methods in the European Prospective Investigation of Cancer in Norfolk. Public Health Nutr 2001;4:847-58.
54. Welch A, McTaggart A, Mulligan A, et al. DINER (Data into Nutrients for Epidemiological Research)—a new data-entry program for nutritional analysis in the EPIC-Norfolk cohort and the 7-day diary method. Public Health Nutr 2001;4:1253-65.
55. Liggins J, Bluck LJC, Coward WA, Bingham SA. Extraction and quantification of daidzein and genistein in food. Anal Biochem 1998;264:1-7.
56. Liggins J, Bluck LJC, Runswick S, Atkinson C, Coward WA, Bingham SA. Daidzein and genistein content of fruits and nuts. J Nutr Biochem 2000;11:326-31.
57. Liggins J, Bluck LJC, Runswick S, Atkinson C, Coward WA, Bingham SA. Daidzein and genistein contents of vegetables. Br J Nutr 2000; 84:717-25.
58. Liggins J, Mulligan A, Runswick S, Bingham SA. Daidzein and genistein content of cereals. Eur J Clin Nutr 2002;56:961-6.
59. Kiely M, Faughnan MS, Wähälä K, Brants H, Mulligan A. Phytoestrogen levels in foods: the design and construction of the VENUS database. Br J Nutr 2003;89:S19-23.
60. van Erp-Baart M-AJ, Brants HA, Kiely M, et al. Isoflavone intake in four different European countries: the VENUS approach. Br J Nutr 2003;89:S25-30.
61. De Kleijn MJJ, Van der Schouw YT, Wilson PWF, et al. Intake of dietary phytoestrogens is low in postmenopausal women in the United States: the Framingham study. J Nutr 2001;131:1826-32.
62. Kelly GE, Joannou GE, Reeder AY, Nelson C, Waring MA. The variable metabolic response to dietary isoflavones in humans. Proc Soc Exp Biol Med 1995;208:40-3.
63. Setchell KDR, Brown NM, Desai P, et al. Bioavailability, disposition, and dose-response effects of soy isoflavones when consumed by healthy women at physiologically typical dietary intakes. J Nutr 2003; 133:1027-35.
64. Horn-Ross PL, Barnes S, Lee M, et al. Assessing phytoestrogen exposure in epidemiologic studies: development of a database (United States). Cancer Causes & Control 2000;11:289-98.
65. Lampe, J. Isoflavonoid and lignan phytoestrogens as dietary biomarkers. J Nutr 2003;133:956S-64S.
66. Lampe JW, Karr S, Hutchins A, Slavin J. Urinary equol excretion with a soy challenge: influence of habitual diet. Proc Soc Exp Biol Med 1998;217:335-9.
67. Rowland IR, Wiseman H, Sanders TAB, Adlercreutz H, Bowey EA. Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. Nutr Cancer 2000;36:27-32.
68. Franke AA, Custer LJ, Wilkens LR, et al. Liquid chromatographic-photodiode array mass spectrometric analysis of dietary phytoestrogens from human urine and blood. J Chromatogr B 2002;777: 45-59.