

Soy Isoflavone Supplementation for Breast Cancer Risk Reduction: A Randomized Phase II Trial

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Abstract

Soy isoflavone consumption may protect against breast cancer development. We conducted a phase IIB trial of soy isoflavone supplementation to examine its effect on breast epithelial proliferation and other biomarkers in the healthy high-risk breast. One hundred and twenty-six consented women underwent a random fine-needle aspiration (rFNA); those with 4,000 or more epithelial cells were randomized to a double-blind 6-month intervention of mixed soy isoflavones (PTIG-2535) or placebo, followed by repeat rFNA. Cells were examined for Ki-67 labeling index and atypia. Expression of 28 genes related to proliferation, apoptosis, and estrogenic effect was measured using quantitative reverse transcriptase PCR. Hormone and protein levels were measured in nipple aspirate fluid (NAF). All statistical tests were two-sided. Ninety-eight women were evaluable for Ki-67 labeling index. In 49 treated women, the median Ki-67 labeling index was 1.18 at entry and 1.12 post intervention, whereas in 49 placebo subjects, it was 0.97 and 0.92 (P for between-group change: 0.32). Menopausal stratification yielded similar results between groups, but within premenopausal soy-treated women, Ki-67 labeling index increased from 1.71 to 2.18 ($P = 0.04$). We saw no treatment effect on cytologic atypia or NAF parameters. There were significant increases in the expression of 14 of 28 genes within the soy, but not the control group, without significant between-group differences. Plasma genistein values showed excellent compliance. A 6-month intervention of mixed soy isoflavones in healthy, high-risk adult Western women did not reduce breast epithelial proliferation, suggesting a lack of efficacy for breast cancer prevention and a possible adverse effect in premenopausal women. *Cancer Prev Res*; 5(2); 309–19. ©2012 AACR.

Introduction

The primary prevention of breast cancer currently rests on the selective estrogen receptor modulators (SERM) tamoxifen (1) and, for postmenopausal women, raloxifene (2). However, toxicity concerns have rendered these

generally unacceptable to healthy women (3–5). Dietary soy, or components of it such as genistein, may contribute to the lower breast cancer incidence seen in populations with high soy consumption, as shown in several epidemiologic investigations (6, 7). Recent studies have also suggested a favorable effect on breast cancer survival (8). However, the beneficial effect of soy consumption on breast cancer risk may derive from exposure early in life, and the introduction of soy isoflavones into the diets of adult Western women may have minimal impact (9). Thus, well-designed prospective intervention studies are needed to support the epidemiologic data and allay concerns about a possible harmful pro-estrogenic effect of soy supplements, as suggested by several rodent studies (10, 11). Because commercially available soy isoflavone supplements are being widely consumed by women of all age groups for a variety of reasons, it is important to know whether soy isoflavones induce proliferation in the healthy breast. Furthermore, observation of an antiproliferative effect would be grounds for wider investigation of soy isoflavones as breast cancer preventive agents in adult Western populations.

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We undertook a phase IIB placebo-controlled randomized trial of a mixed isoflavone compound in healthy, high-risk women to test the hypothesis that soy isoflavone supplementation for 6 months will decrease breast epithelial cell proliferation, measured as the Ki-67 labeling index. This is the first report of a uniform high-risk population undergoing a well-defined soy isoflavone intervention with breast tissue biomarker analyses prior to and following the intervention.

Methods

Study design

The study population consisted of healthy, nonpregnant, and nonlactating women at increased risk for breast cancer or women with a history of unilateral minimal risk breast cancer (Tis, or T1a-b, N0 breast cancer, when only the unaffected breast was studied). Subjects were recruited from the Lynn Sage Breast Center and the Bluhm Family Program for Breast Cancer Early Detection and Prevention of Northwestern Memorial Hospital, Chicago, IL. The study protocol was approved by the Institutional Review Board of Northwestern University, and all subjects signed a document of informed consent. Eligible women were 25 to 55 years in age, with a 5-year Gail or Claus model risk estimate $\geq 1.66\%$ for women older than 40 years, $\geq 1.0\%$ for those aged between 30 and 39, and $\geq 0.1\%$ for women aged between 20 and 29. Adequate bone marrow, liver, kidney, and thyroid function was required. Participants were asked to avoid soy-containing foods and supplements, hormonal contraceptives, and hormone therapy and kept a 6-month diary of ingested foods, herbs, supplements, and medications.

Participants underwent a 2-week washout period where they avoided all soy foods, followed by the baseline study visit where breast epithelium was sampled by random fine-needle aspiration (rFNA); nipple aspiration fluid (NAF) and peripheral blood was also collected. NAF samples were pooled if obtained from both breasts. The timing of the rFNA was in mid-luteal phase, predicted by the date of the last period and the usual length of the cycle. This was confirmed by the date of the next menstrual period and the serum progesterone concentrations. The rFNA was conducted as described by Fabian and colleagues (12); samples from both breasts were pooled. Subjects with an epithelial yield of 4,000 or more cells were randomized 1:1 in a double-blind fashion to either one capsule per day of mixed soy isoflavones, or placebo, for a period of 6 months, followed by repeat rFNA, NAF, and blood collection. Stratification factors included menopausal status and history of unilateral cancer. Participants were designated postmenopausal if plasma follicle-stimulating hormone (FSH) > 30 mIU/mL, estradiol < 30 pg/mL, and progesterone < 1 pg/mL with no menstrual period within 6 months. The study agent, PTIG-2535, contained 150 mg genistein, 74 mg daidzein, and 11 mg glycitein. PTIG-2535 and matched placebo pills were supplied by the Division of Cancer Prevention, National Cancer Institute, Bethesda,

MD. Women were declared noncompliant if they consumed less than 80% of the dose based on pill counts or if they had a lapse of 1 week or more during the last month of intervention.

Study endpoints

The primary endpoint was breast epithelial cell proliferation. Secondary endpoints included cytomorphologic assessment of atypia and spectral imaging analysis of atypical features in epithelial cells (13). The expression of a panel of 28 genes (selected on the basis of estrogen or genistein responsiveness, or because of an association with atypia in the breast) was measured in rFNA samples using quantitative reverse transcriptase PCR (qRT-PCR). The breast endocrine environment was measured in NAF samples: estradiol, cathepsin D, insulin-like growth factor-I (IGF-I), and epidermal growth factor (EGF). Plasma samples were assayed for genistein, equol, estradiol, progesterone, sex-hormone-binding globulin (SHBG), and FSH.

Laboratory methods

Cytology and Ki-67 assessment. The rFNA samples were rinsed into cold Cytolyte on ice and centrifuged immediately; the cell pellet was resuspended in 1 mL Cytolyte and split into aliquots for RNA extraction (-fifth) and cytology (one-fifth). RNA aliquots were resuspended in 1 mL of TRIzol and stored at -80°C . Cytology aliquots were pre-filtered through a 20- μm nylon net filter (catalog no. NY200470; Millipore); ThinPrep slides were prepared for Papanicolaou staining and immunohistochemistry. Immunostaining for Ki-67 was conducted with mouse monoclonal antibody Clone MIB-1 (M7240; Dako Corp), in batches containing pre- and postintervention samples from each subject (14). Each run included a reference sample obtained by pooling of several rFNA aspirations of prophylactic mastectomy specimens and a negative control. Assessment of Ki-67 staining was by manual touch counts of a minimum of 500 epithelial cells on digitized images, using Metamorph software; 10% of samples were blindly recounted by the same observer (D. Ivancic) and 20% of samples were assessed by a different observer using image analysis which involved standardized automatic acquisition (TissueFAXS 1.2.4 software; TissueGnostics) and a motor stage (Märzhäuser). The intraobserver correlation was 0.88 and the interobserver correlation was 0.86. The mean Ki-67 labeling index for the positive control slide was 4.27 (range, 3.99–5.10, SD: 0.42). Cytologic atypia evaluation was conducted on Papanicolaou stained ThinPrep slides using standard criteria (15, 16), which were also used for spectral spatial imaging. Cell clusters were used to generate image stacks with the Nuance LCTF-based imaging system (CRI Inc). To build the algorithmic model, image stacks were analyzed using a neural network-based artificial intelligence system now distributed commercially as the InForm system. Manual painting of atypical (red) and benign (green) features was followed by application of a diagnostic algorithmic previously developed and tested in benign versus malignant breast cytologic samples (17); the

image data were collected as percentage of pixels assigned as "atypical."

RNA analyses. Total mRNA was extracted from rFNA samples using TRIzol (Sigma-Aldrich) and purified using the RNeasy Plus Micro Kit (# 74034; Qiagen). A total of 100 ng of RNA was reverse transcribed using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Nine participants whose clinical samples did not yield 100 ng RNA were not analyzed. Amplicons of interest were linearly amplified using the TaqMan PreAmp Master Mix Kit (Applied Biosystems) with 10 cycles of amplification. We selected 28 genes including 14 genes reported as the molecular targets of genistein *in vitro* (18, 19), 9 estrogen receptor (ER α)-related genes identified in benign breast samples (our unpublished data) and 5 genes associated with breast epithelial atypia (20). Two housekeeping genes (*GAPDH* and *HPRT1*) were chosen for normalization. TaqMan low-density gene expression assays (TLDA) were preloaded in 384-well microfluidic cards (each gene in triplicate) from Applied Biosystems. Assays were designed with small amplicons (<100 bp) to enhance detection sensitivity. Real-time PCR reactions were carried out in an Applied Biosystems 7900HT machine. For each gene of interest, expression levels were normalized to the average expression of *GAPDH* and *HPRT1*. Seven samples with sufficient cDNA to allow qRT-PCR without amplification were checked against the results from the postamplification TLDA assays to confirm linear amplification; the values for amplified and unamplified cDNA were highly correlated ($R^2 = 0.95$); the plot resulting from this comparison is shown in Supplementary Fig. S1.

Plasma hormone assays. Plasma was assayed for estradiol, progesterone, FSH, and SHBG. Radioimmunoassay (RIA) kits were purchased from Diagnostic Systems Laboratories (DSL) for estradiol and progesterone quantification. An enzyme immunoassay (EIA) purchased from Alpco Diagnostics was used for FSH quantification. An Iso-Data 20/20 Series gamma counter was used for measurements in the RIAs, and a BIO-TEK Synergy HT plate reader was used for the measurements in the EIAs.

Plasma genistein and equol assays. High-pressure liquid chromatography (HPLC) analysis with electrochemical detection of plasma soy isoflavones was carried out using the procedure of Gamache and Acworth with slight modifications (21;22). Genistein and equol concentrations were measured taking into account the recovery of an estriol-glucuronide internal control at a concentration of 2 nmol/mL (973 ng/mL; ref. 23). Estriol recovery was 75% [coefficient of variance (CV), 15.5%] in this series of 190 plasma samples, excluding 5 outliers. Estriol serum levels in pre- and postmenopausal women are in the range of 6 to 12 pg/mL, therefore endogenous estriol was not a concern. The CVs for the positive genistein, equol, and estriol spiked methanol controls were 18%, 9%, and 7%, respectively. The sensitivity of this assay for genistein and equol is approximately 3 ng/mL.

NAF hormone and protein assays. NAF volume was measured in calibrated capillary tubes and diluted in PBS.

Estrogens were extracted into ethyl acetate:hexane (3:2), and the extract was fractionated by HPLC on a C18 column as described previously (24). The recovery of E2 averaged 78.3%, with a lower limit of detection of 6.25 pg/mL. The intra- and interassay %CVs were: E2, 4.89% and 6.55%; E1, 5.38% and 6.82%; EGF, 5.38% and 20.2%; cathepsin D, 8.63% and 36.6%; and IGF-I, 6.68% (BCF does not have detectable IGF-I for calculation of interassay variation). Modified RIA kits from DSL were used for estradiol quantification (25). Cathepsin D and EGF were measured in the aqueous fraction with EIA kits from Calbiochem and Alpco Diagnostics, respectively. IGF-I was measured in the aqueous fraction with an RIA from Alpco Diagnostics.

NAF isoflavones. A total of 150 μ L diluted NAF was mixed with 15 μ L triply labeled 13 C-standards of daidzein, genistein, and equol (purchased from the University of St. Andrews, Scotland, UK), incubated with β -glucuronidase and arylsulfatase. This mixture was extracted with methyl tertiary-butyl ether and analyzed by liquid chromatography/mass spectrometry (LC/MS) using a Gemini C18 analytical column (150 \times 2.0; 5 μ m; Phenomenex) with the following linear gradient of A = methanol/acetonitrile (1:1) and B = water at 0.2 mL/minute (%B): 40% to 60% in 2.5 minutes, hold at 60% for 5.5 minutes and equilibrate at 40% for 2 minutes before subsequent injections. Electrospray ionization followed by high-accuracy orbitrap mass spectrometry (model Exactive, ThermoFisher) in negative mode was applied for all analytes according to the published method (26). The lower limits of detection for daidzein, genistein, and equol were 0.2, 0.5, and 1.2 ng/mL aqueous fraction. The intra- and interassay %CVs were 9% to 13% for all analytes in a concentration range of 5 to 30 ng/mL.

Statistical methods

We planned to accrue 150 women and randomize 120, expecting that 80% of subjects would yield sufficient epithelial cells for analysis ($\geq 4,000$ cells). With a 28% dropout rate (including women who had insufficient cells for analysis the 6-month time point), we planned a total of 90 women (45 per group) for final analysis. We estimated a median postintervention decrease in the primary endpoint (Ki-67 labeling index of epithelial cells) of 1.5% in the soy group, compared with a median change of zero in the control group. Assuming an SD of 1.5% to 2%, this would provide more than 90% power with 45 subjects per group. Interim analyses were planned to identify evidence for a systemic estrogenic effect of the soy isoflavone supplement, defined as an increase in the 1-month plasma in SHBG of 1.5 times the baseline level.

The baseline demographic characteristics between treatment and control groups were compared using the Wilcoxon rank-sum test for continuous variables and Fisher exact test for categorical variables. Analyses of cellular parameters were adjusted for cell number. The effects of treatment were assessed within groups (month 6 – baseline) using the signed-rank test and

between groups (treated difference – control difference) using the Wilcoxon rank-sum test. Women with plasma equol concentrations >5 ng/mL were designated as equol producers (27). For NAF data, because there was a substantial proportion of nondetectable values, we first calculated month 6 minus baseline changes and then categorized these changes into tertiles. We then compared frequencies of subjects in each tertile between groups using Fisher exact test. For gene expression data, we obtained cycle threshold (C_t) values from the PCR experiments; C_t outliers within triplicates [determined using the Grubbs (1950) method] were omitted (28). C_t values were averaged across triplicates by subject, gene, and visit. Genes were normalized by subtracting the mean of the housekeeping genes *GAPDH* and *HPRT1* for each subject, gene, and visit (ΔC_t). The normalized baseline, month 6, and month 6 minus baseline values ($\Delta\Delta C_t$) were exponentiated by a negative power of 2. The means, SDs, and 95% CIs were calculated for the exponentiated data. The month 6 minus baseline differences between groups were tested using the unpaired *t* test, whereas differences within groups were tested using the paired *t* test. We adjusted *P* values from these tests via the Benjamini-Hochberg approach (29). We also conducted a global analysis to examine whether there was an overall difference among treatment and menopausal groups in month 6 minus baseline changes across all 30 genes combined. Global tests here were based on an ANOVA. To examine the similarity among the samples on gene expression profiles, a clustering analysis was conducted using Cluster v2.11 and TreeView v1.6 from Michael Eisen. All statistical tests were 2-sided.

Results

Of the 150 women consented, 138 underwent the entry rFNA procedure, with 12 (8.7%) yielding insufficient cells, so that 126 subjects were randomized. Of these, 98 (77.8%) had more than 4,000 epithelial cells in rFNA samples pre- and postintervention, met the criteria for compliance, and were evaluable for the primary endpoint of Ki-67 labeling of epithelial cells at both time points. The CONSORT diagram is shown in Fig. 1, and Table 1 shows the evaluable participant characteristics. Mid-luteal phase timing of the rFNA was achieved at both time points in 43 of 53 (81%) premenopausal women. In 10 women, luteal phase timing could not be confirmed because the cycles had become irregular. Because results were similar in analyses restricted to the 43 women who were in luteal phase at both time points and in all 53 premenopausal women, we have presented results for all premenopausal women. Compliance to the study regimen was excellent among the 98 women included in the final analysis, as shown in Table 2. The median plasma genistein levels were 156 ng/mL in postmenopausal women and 205 ng/mL in premenopausal women in the treated group, compared with 0 in the control group. The median plasma concentration of FSH and SHBG, and ratio of estradiol to SHBG, did not change

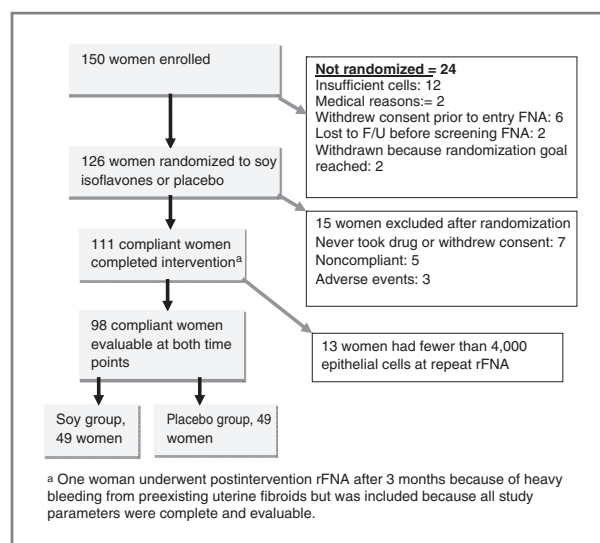


Figure 1. CONSORT diagram: retention of participants through the study.

following intervention, in both pre- and postmenopausal women (see Table 2).

The results related to proliferation and cytologic features of the epithelial cells are shown in Table 3. The mean epithelial cell yield at baseline was 40,030 and was 47,867 post intervention. As expected, the baseline Ki-67 labeling index was significantly higher in premenopausal than in postmenopausal women (1.79 vs. 0.76, $P < 0.001$) and was higher in samples obtained in luteal phase than in follicular phase (1.95 vs. 1.26, $P < 0.04$). In the control group, the Ki-67 labeling index was concordant between entry and 6-month samples, with a Pearson $R^2 = 0.61$ ($P < 0.0001$). The change in Ki-67 labeling index (i.e., month 6 – baseline values) was similar between the soy and placebo groups in the entire study population. In contrast, following menopausal stratification, we observed a statistically significant increase in Ki-67 labeling index from baseline to postintervention within the premenopausal soy-treated women (1.71 vs. 2.18, $P = 0.04$) but not in control premenopausal women (1.90 vs. 1.94, $P = 0.56$). We then compared the median change in Ki-67 labeling index between treated and control premenopausal subjects and found no significant difference (0.19%; interquartile range, -0.46 to 1.07 , $P = 0.31$). Among postmenopausal women, there were no significant differences in Ki-67 labeling, within or between treated and placebo groups, comparing baseline with postintervention values. Notably, the direction of the postintervention change in Ki-67 labeling index was significantly different between pre- and postmenopausal women ($+0.19$ vs. -0.13 , $P = 0.03$). We did find a significant positive relationship between Ki-67 labeling index and cytologic atypia ($P < 0.02$) and with the lifetime Gail risk estimate ($P = 0.005$), despite a significant negative association with age ($P = 0.001$). However, the association between Ki-67 labeling index and epithelial cell number was weak and nonsignificant.

Table 1. Characteristics of evaluable participants

	Soy group (N = 49)	Control group (N = 49)	P
Age (interquartile range), y	48 (43–53)	50 (46–55)	0.27
Race			
African American	4 (8.2%)	6 (12.2%)	0.74
White	45 (91.8%)	43 (87.8%)	
Ethnicity			
Gail risk estimate (5 y)	2.10 (1.75, 3.15)	2.20 (1.80, 2.70)	0.69
Gail risk estimate (lifetime)	19.10 (15.20, 26.50)	17.30 (15.00, 25.80)	0.45
Menopause status at study entry			
Pre	28 (57.1%)	25 (51.0%)	0.69
Post	21 (42.9%)	24 (49.0%)	
Menstrual phase at rFNA			
Follicular at both	2 (7.1%)	4 (16.0%)	0.28
Luteal at both	25 (89.3%)	18 (72.0%)	
Discordant	1 (3.6%)	3 (12.0%)	
Soy stratification			
Premenopausal, no cancer	23 (46.9%)	21 (42.9%)	0.85
Postmenopausal, no cancer	14 (28.6%)	15 (30.5%)	
History of ER ⁻ cancer ^a	6 (12.3%)	4 (8.2%)	
History of ER ⁺ cancer ^a	9 (12.3%)	6 (18.4%)	

^aHistory of unilateral breast cancer with all systemic therapy completed at least 1 year previously, only unaffected breast sampled.

There were no significant between-group changes in morphologic features of the epithelial cells, measured categorically as the presence or absence of cytologic atypia, or as assessed using the Masood score. This was true for all women and for tests stratified by menopausal status (Table 3). Despite a borderline improvement in the post-intervention Masood score within the soy-treated postmenopausal group (from 14 to 13, $P = 0.04$), there was no significant difference between treated and control postmenopausal women. Similarly, the presence of atypical features by spectral-spatial imaging showed that the median fraction of epithelial clusters showing atypical features was similar pre- and postintervention, within and between groups.

Gene expression patterns for the 28 genes evaluated were similar at baseline between treated and control women; with *GAPDH* and *HPRT1* as reference genes, the mean fold expression of the genes of interest was 1.56 for controls and 1.42 for the soy group ($P = 0.28$), with no significant effect of menopausal status ($P = 0.11$). Within the soy group, we observed a significant increase in expression of 14 of 28 genes from baseline to postintervention, with adjusted P values ranging from 0.017 for *BCL-2* to 0.052 for *FAS* (Table 4). These included 7 that had been selected on the basis of genistein response (*BCL-2*, *CDKN1A*, *CDKN2A*, *DDIT3*, *FAS*, *PARP-1*, and *TP53*), 5 that were chosen based on estrogen response (*ESR1*, *FOXA1*, *MYB*, *PGR*, and *SCUBE2*) and 2 that were selected because of an association with breast epithelial atypia (*AR* and *Wnt5B*). In contrast, there were no significant changes in the expression of any of the 28 genes within the control group. The mean fold

change from baseline to 6 months across all 28 genes in the soy-treated women was 1.56 versus 1.25 for control subjects ($P = 0.02$). At the 6-month time point, the mean fold expression (relative to housekeeping genes) in the soy group was 1.66 versus 1.31 for the control subjects ($P = 0.0001$). On examining month 6 minus baseline differences in individual gene expression between soy and placebo groups, 4 genes showed a significantly larger increase in the soy than in the placebo group (*ESR1*, *FAS*, *FOXA1*, and *MYB*) but these increases were not significant following Benjamini–Hochberg adjustment. Detailed results for individual genes within and between groups are shown in Table 4. A heatmap of gene expression patterns of month 6 minus baseline differences in the soy group is shown in Fig. 2. Samples clustered into 2 branches, with overall expression increasing in the first branch (cluster A, B, C, and D) and decreasing in the second branch (cluster E, F, and G). In cluster A and B (20 subjects), the expression of estrogen-responsive and epithelial atypia-associated genes was dramatically stimulated, whereas the expression of genistein target genes was moderately increased. On the contrary, in cluster E and F (15 subjects), the expression of estrogen-responsive genes and epithelial atypia-associated genes was dramatically suppressed, and the expression of genistein target genes was moderately decreased. The samples in cluster C, D, and G (12 subjects) showed intermediate expression pattern between cluster A/B and cluster E/F.

NAF collection was attempted on all women and was successful in 46 women at both time points (26 in the soy group and 20 in the control group). The tertile distribution of postintervention change in NAF volume, estradiol, and

Table 2. Plasma genistein and endocrine parameters in treatment and placebo groups

Parameter	Soy group (n = 49)			Placebo group (n = 49)			Between-group	
	N	Entry	Six mo	Entry	Six mo	Difference	P	
Plasma genistein in units (median and interquartile range), ng/mL								
All patients	97	0 (0-0)	174 (113-377)	0 (0-0)	0 (0-0)	174 (113-377)	<0.0001	
Postmenopausal	44	0 (0-0)	143 (19-383)	0 (0-0)	0 (0-0)	143 (19-383)	<0.0001	
Premenopausal	53	0 (0-0)	205 (124-374)	0 (0-0)	0 (0-0)	205 (124-374)	<0.0001	
Median plasma estradiol (median and interquartile range), pg/mL								
All patients	97	26.12 (12.73-54.59)	26.01 (15.68-59.16)	17.01 (10.18-38.52)	21.74 (13.46-55.89)	4.12 (-6.52 to 17.91)	0.77	
Postmenopausal	44	11.54 (6.61-16.62)	16.46 (12.70-20.59)	10.31 (9.33-16.62)	14.04 (12.00-19.20)	2.90 (-1.54 to 9.01)	0.36	
Premenopausal	53	45.29 (28.71-72.35)	47.82 (26.63-72.93)	36.32 (20.38-70.43)	51.94 (43.38-72.92)	8.94-19.86 to 33.42)	0.57	
Median SHBG (median and interquartile range), nmol/L								
All patients	97	61.25 (45.52-104.35)	71.30 (47.22-97.34)	85.84 (55.26-118.71)	78.47 (55.84-111.85)	-5.34 (-18.82 to 15.11)	0.43	
Postmenopausal	44	78.01 (54.98-100.97)	72.94 (56.59-119.38)	103.37 (58.27-122.09)	86.05 (57.19-121.63)	-4.98 (-17.82 to 15.28)	0.57	
Premenopausal	53	55.13 (39.56-105.54)	63.21 (42.85-91.02)	77.28 (51.79-118.71)	74.65 (53.68-111.85)	-5.34 (-21.44 to 9.45)	0.56	
Median ratio of estradiol to SHBG (median and interquartile range)								
All patients	97	0.32 (0.18-0.83)	0.37 (0.20-0.87)	0.24 (0.12-0.51)	0.40 (0.12-0.80)	0.08 (-0.05 to 0.26)	0.48	
Postmenopausal	44	0.16 (0.10-0.21)	0.20 (0.13-0.34)	0.14 (0.08-0.19)	0.18 (0.09-0.36)	0.03 (-0.02 to 0.11)	0.72	
Premenopausal	53	0.73 (0.37-1.31)	0.67 (0.37-1.15)	0.46 (0.30-0.77)	0.80 (0.50-1.10)	0.15 (-0.23 to 0.58)	0.28	
Median FSH (median and interquartile range), mIU/mL								
All patients	97	10.90 (5.53-69.76)	17.43 (5.17-84.79)	34.78 (4.24-72.62)	49.67 (5.80-77.77)	2.75 (-4.15 to 11.66)	0.45	
Postmenopausal	44	71.72 (48.73-90.67)	85.63 (76.52-93.54)	70.85 (50.23-96.13)	77.40 (65.82-95.68)	6.75 (-4.09 to 26.87)	0.72	
Premenopausal	53	5.73 (2.46-8.30)	6.01 (0.01-9.64)	4.24 (1.50-12.92)	5.80 (0.01-20.97)	0.00 (-4.15 to 5.87)	0.80	

protein concentrations (total protein, EGF, cathepsin D, IGF-I) was compared between groups. Numerical values for each parameter are shown in Supplementary Table S1. We found no significant differences in the tertiles of changes in any of these parameters between soy and placebo groups. However, the median NAF genistein concentration was significantly different, being 64.2 ng/mL in the treated

women, and 6.2 ng/mL in the placebo group ($P < 0.0001$). Within the soy-treated cohort, there was no correlation between NAF and plasma genistein concentrations at the 6-month time point ($P = 0.54$), or between NAF genistein or daidzein values and Ki-67 labeling index in breast epithelial cells at 6 months ($R^2 = 0.02$ and $P = 0.32$ for genistein, $R^2 = 0.01$ and $P = 0.56$ for daidzein).

Table 3. Cellular parameters in treatment and placebo groups

Parameter	Soy group (n = 49)			Placebo group (n = 49)			Between group		
	Entry	Six mo	P	Entry	Six mo	P	Difference	P	
Median Ki-67 labeling index (interquartile range)									
All subjects	98	1.17 (0.66-1.93)	1.09 (0.75-2.33)	0.82	0.97 (0.70-1.90)	0.92 (0.59-1.96)	0.14	-0.03 (-0.42 to 0.08)	0.24
Postmenopausal	45	0.63 (0.52-1.08)	0.77 (0.35-0.94)	0.56	0.70 (0.57-1.07)	0.63 (0.42-0.98)	0.22	-0.12 (-0.37 to 0.23)	0.73
Premenopausal	53	1.71 (1.12-2.35)	2.18 (1.18-3.04)	0.04	1.90 (0.88-2.33)	1.94 (0.92-2.55)	0.56	0.19 (-0.46 to 1.07)	0.31
Proportion of women with atypical cytology									
All subjects	98	42.9%	53.1%	0.42	40.8%	53.1%	0.31	-2.1%	0.83
Postmenopausal	45	33.3%	23.8%	0.73	33.3%	33.3%	0.99	-9.5%	0.72
Premenopausal	53	50.0%	75.0%	0.10	48.0%	72.0%	0.15	1.0%	0.99
Median Masood score (interquartile range)									
All subjects	98	14.0 (13.0-15.0)	14.0 (12.0-15.0)	0.68	13.0 (13.0-15.0)	14.0 (13.0-15.0)	0.37	0.0 (-1.0 to 1.0)	0.64
Postmenopausal	45	14.0 (13.0-15.0)	13.0 (12.0-14.0)	0.04	13.0 (13.0-14.5)	13.0 (12.0-15.0)	0.32	0.0 (-1.0 to 0.0)	0.15
Premenopausal	53	14.0 (13.0-15.5)	15.0 (14.0-16.0)	0.33	14.0 (13.0-16.0)	15.0 (14.0-16.0)	0.13	1.0 (-1.0 to 2.0)	0.41
Atypical features on spectral imaging (interquartile range)									
All subjects	94	0.42 (0.15-0.60)	0.32 (0.10-0.58)	0.50	0.42 (0.15-0.60)	0.51 (0.19-0.72)	0.63	0.02 (-0.28 to 0.23)	0.47
Postmenopausal	43	0.41 (0.10-0.51)	0.38 (0.08-0.56)	0.57	0.40 (0.12-0.56)	0.29 (0.14-0.58)	0.70	0.04 (-0.21 to 0.24)	0.81
Premenopausal	51	0.47 (0.23-0.66)	0.28 (0.10-0.60)	0.29	0.45 (0.18-0.75)	0.62 (0.28-0.77)	0.66	0.00 (-0.30 to 0.21)	0.32

Table 4. Changes in expression of individual genes, month 6 minus baseline values

Gene name	Differences within the soy group		Differences within the control group		<i>P</i> for between-group differences	
	Mean fold change ± SD	Adjusted <i>P</i>	Mean fold change ± SD	Adjusted <i>P</i>	Raw <i>P</i>	Adjusted <i>P</i>
Genistein molecular targets						
<i>BAX</i> -Hs00180269_m1	1.15 ± 0.56	0.123	0.98 ± 0.62	0.874	0.178	0.654
<i>BCL2</i> -Hs99999018_m1	1.34 ± 0.58	0.017	1.24 ± 0.82	0.109	0.499	0.705
<i>BCL3</i> -Hs00180403_m1	1.21 ± 0.79	0.123	1.11 ± 0.99	0.512	0.585	0.705
<i>BIRC5</i> -Hs00153353_m1	1.79 ± 2.8	0.109	1.91 ± 3.62	0.15	0.887	0.887
<i>CCND1</i> -Hs00765553_m1	3.27 ± 8.65	0.123	2.21 ± 3.85	0.101	0.455	0.705
<i>CDKN1A</i> -Hs00355782_m1	1.58 ± 1.4	0.035	1.24 ± 1.28	0.27	0.226	0.654
<i>CDKN2A</i> -Hs99999189_m1	1.43 ± 0.9	0.025	1.26 ± 0.82	0.101	0.349	0.654
<i>DDIT3</i> -Hs01090850_m1	1.37 ± 0.92	0.035	1.17 ± 1.11	0.353	0.342	0.654
<i>FAS</i> -Hs00163653_m1	1.32 ± 0.84	0.052	1.01 ± 0.62	0.954	0.050	0.447
<i>GREB1</i> -Hs00536409_m1	4.39 ± 11.3	0.106	2.98 ± 6.74	0.109	0.469	0.705
<i>NFKB1</i> -Hs00765730_m1	1.14 ± 0.43	0.089	1.05 ± 0.48	0.549	0.315	0.654
<i>PARP1</i> -Hs00911369_g1	1.42 ± 0.79	0.022	1.22 ± 1.02	0.189	0.302	0.654
<i>PTGS2</i> -Hs00153133_m1	1.24 ± 0.8	0.109	1.29 ± 1.22	0.159	0.791	0.844
<i>TP53</i> -Hs01034253_m1	1.29 ± 0.71	0.035	1.21 ± 0.73	0.109	0.595	0.705
Estrogen-responsive genes						
<i>ESR1</i> -Hs00174860_m1	2.96 ± 4.33	0.027	1.46 ± 1.82	0.15	0.034	0.447
<i>ESR2</i> -Hs00230957_m1	2.49 ± 5.25	0.109	1.59 ± 2.51	0.166	0.298	0.654
<i>FOXA1</i> -Hs00270129_m1	2.9 ± 4.64	0.035	1.47 ± 1.76	0.123	0.056	0.447
<i>IGF-1</i> -Hs01547657_m1	2.83 ± 6.74	0.122	1.88 ± 2.23	0.046	0.369	0.654
<i>IGFBP5</i> -Hs01052296_m1	3.2 ± 9.49	0.163	1.63 ± 1.94	0.1	0.282	0.654
<i>MYB</i> -Hs00920568_m1	2.44 ± 3.15	0.027	1.3 ± 1.21	0.152	0.025	0.447
<i>PGR</i> -Hs01556702_m1	3.91 ± 7.58	0.046	12.61 ± 68.36	0.303	0.388	0.654
<i>SCUBE2</i> -Hs00221277_m1	4.03 ± 7.16	0.035	2.51 ± 6.1	0.15	0.278	0.654
<i>TFF1</i> -Hs00907239_m1	14.31 ± 42.9	0.101	27.87 ± 139.5	0.25	0.526	0.705
Breast epithelial atypia-associated genes						
<i>PRLR</i> -Hs00168739_m1	2.38 ± 4.01	0.082	1.6 ± 1.89	0.101	0.244	0.654
<i>AR</i> -Hs00171172_m1	3.06 ± 4.56	0.027	1.76 ± 3.31	0.171	0.122	0.654
<i>FGFR3</i> -Hs00997397_m1	18.3 ± 52.5	0.089	6.33 ± 15.85	0.089	0.146	0.654
<i>NDRG2</i> -Hs00212263_m1	1.53 ± 1.8	0.109	1.33 ± 1.61	0.216	0.579	0.705
<i>WNT5B</i> -Hs00364142_m1	1.92 ± 1.92	0.025	2.35 ± 4.54	0.109	0.555	0.705
Housekeeping genes						
<i>GAPDH</i> -Hs00266705_g1	1.01 ± 0.16	0.697	1.02 ± 0.16	0.354	0.68	0.751
<i>HPRT1</i> -Hs01003267_m1	1.01 ± 0.15	0.601	1 ± 0.15	0.954	0.653	0.746

Plasma equol concentrations at entry were equivalent in the soy and placebo groups (2.7 ng/mL in both groups) but were markedly different following intervention, with the treated women displaying a mean plasma equol concentration of 673 ng/mL, compared with 5.6 ng/mL in the placebo group. Measurable plasma equol was greater than 5 ng/mL in 30 of 48 women (62.5%) who were designated equol producers. When compared with control women, the baseline to 6-month change in equol producers showed no significant differences in Ki-67 labeling index, cytologic atypia, median percent atypical features, or estradiol values (Supplementary Table S2), although within premenopausal equol producers, the median change in

Ki-67 labeling index was +0.77 compared with -0.02 in controls ($P = 0.44$).

Serious adverse events occurred in 7 women (5 in the soy group) whereas on study, all considered unrelated to study drug. These included 2 events related to uterine fibroids (anemia requiring hospitalization in one woman and surgery for symptoms in a second); grade III depression in a patient with a history of bipolar disorder 47 days after the last dose; grade III back pain hospitalized for surgery 24 days after initiation of the study drug; and dyspnea 57 days after drug initiation. One placebo subject developed breast cancer; one soy-treated woman discontinued participation because her thyroid-stimulating hormone levels increased.

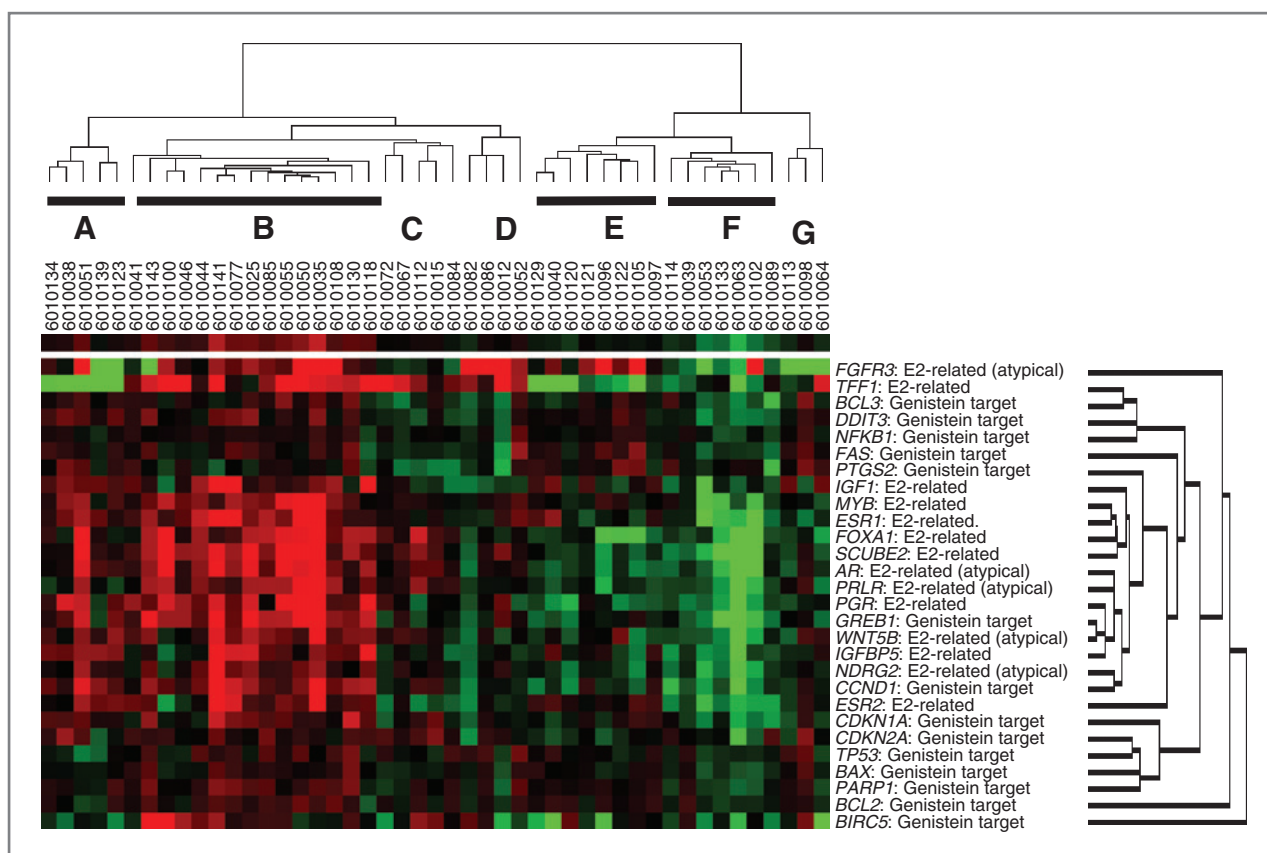


Figure 2. Heatmap of gene expression by TLDA in soy-treated women; clustering by gene group (month 6–baseline differences adjusted by housekeeping genes). Clustering is based on grouping "similar" genes together, where "similarity" here is in turn based on the degree of correlation of expressions levels among genes and among treatment groups. Pearson correlations were used to determine clustering.

Additional data about the distribution of adverse events are presented in Supplementary Table S3.

Discussion

The soy isoflavones are prime dietary candidates for breast cancer prevention, with a wealth of supporting epidemiologic and laboratory data; recent studies provide additional support for a protection against breast cancer causation and relapse (6–8). However, these favorable effects of soy consumption are found mainly in Asian populations (7), with the possible explanation that intake early in life is the key (9). Soy consumption by Western women at risk for breast cancer, and breast cancer survivors, has been deterred by rodent data showing a cancer-promoting effect of soy components (10, 11). Unbiased data on breast outcomes following introduction of soy components into the diets of adult Western women are lacking. We conducted a randomized phase II trial using unconjugated mixed soy isoflavones in a dose that approximates that of the upper quartile of soy-consuming Far-East Asians (8, 30). We used a 2-week washout period prior to randomization and provided all participants with a list of soy-containing foods to avoid during the study period. The median post-

intervention plasma genistein concentrations (174 ng/mL in treated and 0 ng/mL in placebo women) show that our participants adhered well to the study regimen.

We found no significant favorable effect on the primary endpoint of epithelial cell proliferation, as measured by Ki-67 labeling. However, among treated premenopausal women, there was a relative increase of 27% in the postintervention Ki-67 labeling index following soy supplementation which was statistically significant (from 1.71 to 2.18, $P = 0.04$). In contrast, in the premenopausal placebo group, the relative postintervention increase in Ki-67 labeling index was a nonsignificant 2%. However, the postintervention change in Ki-67 labeling index between treated and placebo premenopausal women was not significantly different (relative change 27% vs. 2%, $P = 0.31$). Among postmenopausal women, all Ki-67 comparisons were entirely null. Of interest, the effect of soy on Ki-67 labeling index differed by menopausal status, with a median decrease of 0.13% in the postmenopausal treated group, whereas in premenopausal women, it increased by 0.19% ($P = 0.03$).

The reason for the apparent stimulatory effect upon breast epithelial cell proliferation in premenopausal women is not clear, but a proestrogenic effect of soy is suspect, possibly including an interaction with progesterone, as the

great majority of our premenopausal samples were obtained in luteal phase. In premenopausal treated women, median NAF estradiol content was 116.4 at baseline and 206.3 postintervention; this was not statistically significant but may help to explain the higher Ki-67 labeling index in premenopausal women. A previous study of 2 to 4 weeks of soy isoflavone supplementation in premenopausal women showed no effect on Ki-67 labeling of benign breast tissue, but the tissue assessment was conducted only postintervention (31). Our results for postmenopausal women are in agreement with studies of soy supplementation in oophorectomized macaque monkeys, which failed to show an effect on mammary gland proliferation (32, 33).

We used careful quality control for the measurement of the primary endpoint (Ki-67 labeling index); we counted a mean of 1,593 cells; the intra- and interobserver correlations were high (0.88 and 0.86, respectively) and a positive control sample from pooled *ex vivo* aspirations of prophylactic mastectomy specimens was included in each batch, with excellent batch-to-batch concordance of Ki-67 labeling index. We did not restrict entry to women with atypical cytology, a strategy that has been proposed to ensure that women entering phase II prevention trials have high starting Ki-67. However, the power of finding a difference between groups is driven by the size of the difference and the variation of the data; we observed a similar interquartile range in premenopausal women (baseline median Ki-67 labeling index, 1.85; interquartile range, 0.99–2.33) and in postmenopausal women (baseline median Ki-67 labeling index, 0.79; interquartile range, 0.55–1.08), and our interquartile ranges are smaller than other studies (34). Thus, it is unlikely that a higher starting Ki-67 labeling index would have changed the outcome of our study; but it would have rendered accrual more challenging. Of note, the Masood score at entry in our study population was 13 or greater, the higher end of the hyperplasia without atypia range (11–14).

Among pre- and postmenopausal women, we did not observe any significant changes in cytologic atypia, Masood score, or spectral imaging.

The study plan included an analysis of expression of a panel of 28 genes, pre- and postintervention, selected on the basis of published expression profiles in genistein-treated breast cancer cells, genes involved in estrogen response, and those associated with the presence of epithelial atypia. We saw no significant change in individual gene expression from baseline to postintervention in the placebo group. However, the treated group did show a soy isoflavone signal, with a significant increase in the expression of 14 of 28 genes as well as a significantly higher global expression at 6 months than in the control subjects ($P = 0.0001$).

The specific genes displaying increased expression within the soy group showed a mixed pattern, with more adverse than beneficial effects. For example, ESR1 expression was increased, suggesting an anti-estrogenic effect, but FOXA1, MYB, PGR, TIFF1, and SCUBE-2 were also increased, suggesting estrogenicity. On the cluster trees among the genes, there was a suggestion that the responses to genistein treatment varied among individuals, and most genistein

target genes tended to cluster together on the basis of similar expression patterns. While the exact molecular consequences of change in expression of each gene are undetermined, the stimulation of estrogen-responsive genes in an organ where estrogen is known to increase proliferation suggests a connection between soy isoflavones and increased cell proliferation. There are no comparable data on gene expression changes in breast epithelial samples from healthy women following a preventive intervention in the published literature, and the lack of significance in the between-group changes may relate to variability of gene expression over time in the control group. Notably, the low-density arrays included pre- and postintervention samples of the same subject in the same array, and all PCR reactions were run at the end of the study within a single 3-week period.

We looked for signs of systemic estrogenicity (FSH, SHBG, and estradiol; refs. 35, 36). Estrogenic feedback will also decrease the plasma concentration of estradiol in postmenopausal women (37). None of these effects were observed in response to soy ingestion, suggesting that this combination of soy isoflavones does not cause a systemic estrogenic effect. Alternatively, breast epithelial gene expression and proliferation response may be a more sensitive indicator than these systemic measures.

Finally, although soy isoflavones were reliably detectable in the NAF of women in the soy group, there was no relationship between the presence of genistein in the NAF samples and Ki-67 indices in the breast. These analyses were limited as sufficient NAF yield at baseline and postintervention was achieved in 46 (47%) of women. A previous study suggested a pro-estrogenic effect on the breast based on pre- and postintervention measurements of TIFF1 in NAF samples (30). We did not measure TIFF1, but a number of other estrogen-related proteins (cathepsin D, EGF, and IGF-I) did not change significantly between groups following intervention.

We conducted exploratory analyses focusing on the subset of women who displayed a plasma equol concentration of >5 ng/mL following intervention (27). There was a nonsignificant suggestion of a proliferative response in soy-treated premenopausal equol producers, but our analyses were not powered for the subset of equol producers.

Notably, while soy isoflavone supplementation did not produce favorable biomarker modulation in the breast in the current study, the same agent in the same dose and schedule has produced favorable modulation of a different biomarker (MMP2) in a prostate cancer trial (38). This highlights the potential for organ specificity of preventive agents. Our study also has important differences from the epidemiologic data: dietary soy consumption occurs in smaller doses throughout the day, so that divided doses may have mimicked this pattern more closely. Second, we used a processed supplement, whereas the epidemiologic studies of soy intake have examined intake of whole soy foods. Third, soy exposure early in life may be necessary for beneficial effects (39). Thus, future studies of processed soy supplements for breast cancer protection do not seem

warranted, but investigations of soy food intake, particularly early in life are reasonable.

Disclosure of Potential Conflicts of Interest

The authors had full responsibility for the design of the study, the collection of the data, the analysis and interpretation of the data, the decision to submit the manuscript for publication, and the writing of the manuscript. No potential conflicts of interest were disclosed.

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References

- Fisher B, Costantino JP, Wickerham DL, Cecchini RS, Cronin WM, Robidoux A, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* 2005;97:1652–62.
- Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* 2006;295:2727–41.
- Port ER, Montgomery LL, Heerd AS, Borgen PI. Patient reluctance toward tamoxifen use for breast cancer primary prevention. *Ann Surg Oncol* 2001;8:580–5.
- Tchou J, Hou N, Rademaker A, Jordan VC, Morrow M. Acceptance of tamoxifen chemoprevention by physicians and women at risk. *Cancer* 2004;100:1800–6.
- Yen TW, Hunt KK, Mirza NQ, Thomas ES, Singletary SE, Babiera GV, et al. Physician recommendations regarding tamoxifen and patient utilization of tamoxifen after surgery for ductal carcinoma *in situ*. *Cancer* 2004;100:942–9.
- Trock BJ, Hilakivi-Clarke L, Clarke R. Meta-analysis of soy intake and breast cancer risk. *J Natl Cancer Inst* 2006;98:459–71.
- Dong JY, Qin LQ. Soy isoflavones consumption and risk of breast cancer incidence or recurrence: a meta-analysis of prospective studies. *Breast Cancer Res Treat* 2011;125:315–23.
- Shu XO, Zheng Y, Cai H, Gu K, Chen Z, Zheng W, et al. Soy food intake and breast cancer survival. *JAMA* 2009;302:2437–43.
- Messina M, Hilakivi-Clarke L. Early intake appears to be the key to the proposed protective effects of soy intake against breast cancer. *Nutr Cancer* 2009;61:792–8.
- Allred CD, Ju YH, Allred KF, Chang J, Helferich WG. Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein. *Carcinogenesis* 2001;22:1667–73.
- Allred CD, Allred KF, Ju YH, Clausen LM, Doerge DR, Schantz SL, et al. Dietary genistein results in larger MNU-induced, estrogen-dependent mammary tumors following ovariectomy of Sprague-Dawley rats. *Carcinogenesis* 2004;25:211–8.
- Fabian CJ, Kimler BF, Zalles CM, Klemp JR, Kamel S, Zeiger S, et al. Short-term breast cancer prediction by random periareolar fine-needle aspiration cytology and the Gail risk model. *J Natl Cancer Inst* 2000;92:1217–27.
- Mansoor I, Zalles C, Zahid F, Gossage K, Levenson RM, Rimm DL. Fine-needle aspiration of follicular adenoma versus parathyroid adenoma: the utility of multispectral imaging in differentiating lesions with subtle cytomorphologic differences. *Cancer* 2008;114:22–6.
- Khan SA, Lankes HA, Patil DB, Bryk M, Hou N, Ivancic D, et al. Ductal lavage is an inefficient method of biomarker measurement in high-risk women. *Cancer Prev Res* 2009;2:265–73.
- Masood S, Frykberg ER, McLellan GL, Scalapino MC, Mitchum DG, Bullard JB. Prospective evaluation of radiologically directed fine-needle aspiration biopsy of nonpalpable breast lesions. *Cancer* 1990;66:1480–7.
- The uniform approach to breast fine-needle aspiration biopsy. NIH Consensus Development Conference. *Am J Surg* 1997;174:371–85.
- Zalles C, Zalles N, Mahooti S, Zahid F, Khan SA, Rimm DL. Objective spectral-spatial analysis of random periareolar fine needle aspiration of women at high risk for contralateral breast cancer. In: Proceedings of the CTBC-AACR San Antonio Breast Cancer Symposium; 2009 Dec 13; San Antonio, TX. Abstract nr 6001.
- Banerjee S, Li Y, Wang Z, Sarkar FH. Multi-targeted therapy of cancer by genistein. *Cancer Lett* 2008;269:226–42.
- Dip R, Lenz S, Antignac JP, Le BB, Gmuender H, Naegeli H. Global gene expression profiles induced by phytoestrogens in human breast cancer cells. *Endocr Relat Cancer* 2008;15:161–73.
- Ma XJ, Salunga R, Tuggle JT, Gaudet J, Enright E, McQuary P, et al. Gene expression profiles of human breast cancer progression. *Proc Natl Acad Sci U S A* 2003;100:5974–9.
- Gamache PH, Acworth IN. Analysis of phytoestrogens and polyphenols in plasma, tissue, and urine using HPLC with coulometric array detection. *Proc Soc Exp Biol Med* 1998;217:274–80.
- Franke AA, Custer LJ. High-performance liquid chromatographic assay of isoflavonoids and coumestrol from human urine. *J Chromatogr B Biomed Appl* 1994;662:47–60.
- Rotti K, Stevens J, Watson D, Longcope C. Estradiol concentrations in plasma of normal, non-pregnant women. *Steroids* 1975;25:807–16.
- Chatterton RT Jr, Khan SA, Heinz R, Ivancic D, Lee O. Patterns of sex steroid hormones in nipple aspirate fluid during the menstrual cycle and after menopause in relation to serum concentrations. *Cancer Epidemiol Biomarkers Prev* 2010;19:275–9.
- Chatterton RT Jr, Geiger AS, Khan SA, Helenowski IB, Jovanovic BD, Gann PH. Variation in estradiol, estradiol precursors, and estrogen-related products in nipple aspirate fluid from normal premenopausal women. *Cancer Epidemiol Biomarkers Prev* 2004;13:928–35.
- Maskarinec G, Watts K, Kagihara J, Hebshi SM, Franke AA. Urinary isoflavonoid excretion is similar after consuming soya milk and miso soup in Japanese-American women. *Br J Nutr* 2008;100:424–9.
- Setchell KD, Cole SJ. Method of defining equol-producer status and its frequency among vegetarians. *J Nutr* 2006;136:2188–93.
- Grubbs FE. Criteria for testing outlying observations. *Annals of Mathematical Statistics* 1950;21:27–58.
- Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society* 1995;57:289–300.
- Kurahashi N, Iwasaki M, Sasazuki S, Otani T, Inoue M, Tsugane S. Soy product and isoflavone consumption in relation to prostate cancer in Japanese men. *Cancer Epidemiol Biomarkers Prev* 2007;16:538–45.
- Hargreaves DF, Potten CS, Harding C, Shaw LE, Morton MS, Roberts SA, et al. Two-week dietary soy supplementation has an estrogenic effect on normal premenopausal breast. *J Clin Endocrinol Metab* 1999;84:4017–24.
- Wood CE, Hester JM, Appt SE, Geisinger KR, Cline JM. Estrogen effects on epithelial proliferation and benign proliferative lesions in the postmenopausal primate mammary gland. *Lab Invest* 2008;88:938–48.
- Wood CE, Appt SE, Clarkson TB, Franke AA, Lees CJ, Doerge DR, et al. Effects of high-dose soy isoflavones and equol on reproductive tissues in female cynomolgus monkeys. *Biol Reprod* 2006;75:477–86.

34. Khan QJ, Kimler BF, Clark J, Metheny T, Zalles CM, Fabian CJ. Ki-67 expression in benign breast ductal cells obtained by random periar- eolar fine needle aspiration. *Cancer Epidemiol Biomarkers Prev* 2005;14:786–9.
35. Burger HG, Dudley EC, Robertson DM, Dennerstein L. Hormonal changes in the menopause transition. *Recent Prog Horm Res* 2002; 57:257–75.
36. Plymate SR, Moore DE, Cheng CY, Bardin CW, Southworth MB, Levinski MJ. Sex hormone-binding globulin changes during the menstrual cycle. *J Clin Endocrinol Metab* 1985;61:993–6.
37. Duncan AM, Underhill KE, Xu X, Lavalleur J, Phipps WR, Kurzer MS. Modest hormonal effects of soy isoflavones in postmenopausal women. *J Clin Endocrinol Metab* 1999;84:3479–84.
38. Xu L, Ding Y, Catalona WJ, Yang XJ, Anderson WF, Jovanovic B, et al. MEK4 function, genistein treatment, and invasion of human prostate cancer cells. *J Natl Cancer Inst* 2009;101:1141–55.
39. Korde LA, Wu AH, Fears T, Nomura AM, West DW, Kolonel LN, et al. Childhood soy intake and breast cancer risk in Asian American women. *Cancer Epidemiol Biomarkers Prev* 2009;18: 1050–9.