Randomized Phase IIB Trial of the Lignan Secoisolariciresinol Diglucoside in Premenopausal Women at Increased Risk for Development of Breast Cancer

Carol J. Fabian[1], Seema A. Khan[2], Judy E. Garber[3], William C. Dooley[4], Lisa D. Yee[5], Jennifer R. Klemp[1], Jennifer L. Nydegger[1], Kandy R. Powers[1], Amy L. Kreutzjans[1], Carola M. Zalles[6], Trina Metheny[1], Teresa A. Phillips[1], Jinxiang Hu[7], Devin C. Koestler[7], Prabhakar Chalise[7], Nanda Kumar Yellapu[7], Cheryl Jernigan[8], Brian K. Petroff[9], Stephen D. Hursting[10], and Bruce F. Kimler[11]

ABSTRACT

We conducted a multiinstitutional, placebo-controlled phase IIB trial of the lignan secoisolariciresinol diglucoside (SDG) found in flaxseed. Benign breast tissue was acquired by random periareolar fine needle aspiration (RPFNA) from premenopausal women at increased risk for breast cancer. Those with hyperplasia and ≥2% Ki-67 positive cells were eligible for randomization 2:1 to 50 mg SDG/day (Brevail) versus placebo for 12 months with repeat bio-specimen acquisition. The primary endpoint was difference in change in Ki-67 between randomization groups. A total of 180 women were randomized, with 152 ultimately evaluable for the primary endpoint. Median baseline Ki-67 was 4.1% with no difference between arms. Median Ki-67 change was −1.8% in the SDG arm ($P = 0.001$) and −1.2% for placebo ($P = 0.034$); with no significant difference between arms. As menstrual cycle phase affects proliferation, secondary analysis was performed for 117 women who by progesterone levels were in the same phase of the menstrual cycle at baseline and off-study tissue sampling. The significant Ki-67 decrease persisted for SDG (median = −2.2%; $P = 0.002$) but not placebo (median = −1.0%). qRT-PCR was performed on 77 pairs of tissue specimens. Twenty-two had significant ERα gene expression changes (<0.5 or >2.0) with 7 of 10 increases in placebo and 10 of 12 decreases for SDG ($P = 0.028$), and a difference between arms ($P = 0.017$). Adverse event incidence was similar in both groups, with no evidence that 50 mg/day SDG is harmful. Although the proliferation biomarker analysis showed no difference between the treatment group and the placebo, the trial demonstrated use of SDG is tolerable and safe.

Introduction

There is substantial interest in natural products such as lignans for breast cancer risk reduction given the negligible side effects and potential for other health benefits (1, 2). Secoisolariciresinol diglucoside (SDG) is a plant lignan found in many fruits, vegetables, and oily seeds, with highest concentrations in flaxseed (3). SDG is converted by gut bacteria to the bioactive mammalian lignans enterolactone and enterodiol (3). At premenopausal estradiol levels lignans are thought to act as weak estrogen antagonists whereas at postmenopausal estradiol levels lignans act as weak estrogen agonists (4). Both flaxseed and SDG have been found to improve adipokine profiles and insulin sensitivity in animals on a high fat diet or overweight humans with type II diabetes (5–7). Both have been reported to reduce atherosclerosis progression (8, 9), as well as tumor proliferation and estrogen response gene expression in xenograft models (10, 11). In addition to selective estrogen receptor (ER) modulator activity, lignans are thought to have anti-oxidant, anti-inflammatory, and anti-angiogenic properties, making them attractive for breast cancer risk reduction (12).

[1]Department of Internal Medicine, University of Kansas Medical Center, Kansas City, Kansas. [2]Northwestern University, Chicago, Illinois. [3]Dana-Farber Cancer Institute, Boston, Massachusetts. [4]University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma. [5]Ohio State University, Columbus, Ohio. [6]Department of Pathology, Boca Raton Hospital, Boca Raton, Florida. [7]Department of Biostatistics & Data Science, University of Kansas Medical Center, Kansas City, Kansas. [8]University of Kansas Cancer Center, University of Kansas Medical Center, Kansas City, Kansas. [9]Veterinary Diagnostic Laboratory, Michigan State University, Lansing, Michigan. [10]Department of Nutrition, University of North Carolina, Chapel Hill, North Carolina. [11]Department of Radiation Oncology, University of Kansas Medical Center, Kansas City, Kansas.

Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

Current address for L.D. Yee: City of Hope National Medical Center, Duarte, California.

Corresponding Author: Bruce F. Kimler, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160. Phone: 913-588-4523; Fax: 913-588-3679; E-mail: bkimler@kumc.edu

Cancer Prev Res 2020;13:623–34

doi: 10.1158/1940-6207.CAPR-20-0050

In preclinical studies, SDG significantly increased gene and protein levels of the antioxidants HO-1, GSTM1, and NQO1 (13). SDG may also reduce NFκB proinflammatory signaling through inhibition of p65 phosphorylation, as we have demonstrated in a preclinical model (14). Although NFκB is classically activated by oxidative stress and inflammatory cytokines, it can also be activated by receptor activator of NFκB (RANK), which in turn is upregulated via hormonal stimulation and progesterone receptor activation (15, 16). Our studies of a murine basal-like breast cancer model and two human breast cancer cell lines representing ER^+ Luminal A and claudin-low triple negative suggest that SDG downregulates expression of 50% of genes in the NFκB pathway array (14). NFκB is thought to be important in immune cell infiltration into breast stroma and development of breast cancer (17, 18). IL10, which is upregulated in response to stress, is known to reduce NFκB activity (19).

Lignan dietary intakes in the United States are low and have been reported as <1 mg/day. Canada and some northern European countries have per capita intakes of 8 to 10 times that of the United States (9). Observational studies reporting reduced risk of breast cancer in women with highest versus lowest intakes come predominately from Canadian and northern European cohorts (20, 21). Currently, there is no conclusive evidence of an association between self-reported lignan intake and breast cancer (22–26). Given the uneven sensitivity of dietary tools for assessing lignan intake (27), variability of lignan content in food, and conversion to the bioactive mammalian lignans enterolactone and enterodiol, it would seem more logical to assess breast cancer risk as a function of measured systemic mammalian lignans (28–30). Case–control studies measuring blood enterolactone have generally supported a potential role for lignans in breast cancer risk reduction (31, 32). Enterolactone levels in the highest quintile (>34 nmol/L) in a Finnish case–control study was associated with a 62% reduced risk of breast cancer compared with women in the lowest quintile (<3 nmol/L). Risk was reduced for both pre- and postmenopausal women (31).

We performed a single-arm pilot study in premenopausal women at increased risk for breast cancer in which benign breast tissue was harvested in the follicular phase of the menstrual cycle by random periareolar fine needle aspiration (RPFNA) before and after 12 months of 50 mg/day of SDG given as the commercial product Brevail. Our median 12-month enterolactone level of 99 nmol/L suggested that we were achieving mammalian lignan levels similar to the highest quintile in the Finnish case–control study in which an association with reduced breast cancer incidence was observed. In our pilot study, there was a significant decrease in Ki-67 and borderline significant reduction in the proportion of women showing cytologic atypia, but no significant change in IGF-1, bioavailable estradiol or testosterone, or mammographic breast density using the Cumulus program (33). On the basis of these results, we initiated a placebo-controlled multi-institutional trial (**Fig. 1**) with a 2:1 randomization of eligible women to 12 months of SDG (Brevail) or placebo. Our primary endpoint for comparison of randomization arms was change in Ki-67 over time, with a secondary endpoint of change in cytomorphology. Given the lack of change in mammographic density or IGF-1 in the pilot study, these risk biomarkers were not assessed in the multi-institutional randomized trial. However, estradiol and progesterone were measured as hormone levels may influence proliferation. Change in targeted gene expression in benign breast tissue was explored in archived specimens.

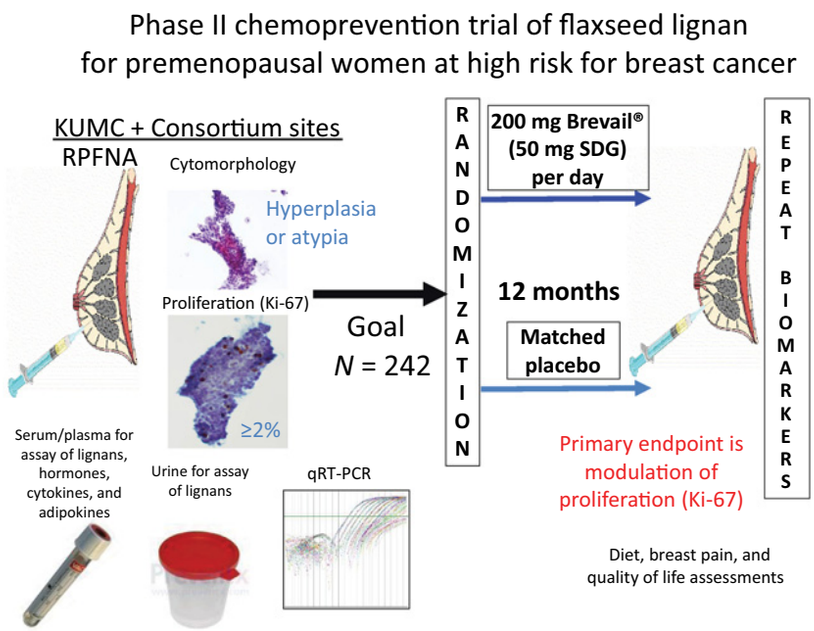

Figure 1.
Schema for phase IIB chemoprevention trial of flaxseed lignan in premenopausal women at high risk for development of breast cancer.

Materials and Methods

Eligibility for study

Premenopausal women age 21 to 49, and BMI <40 kg/m^2 were eligible for tissue screening by RPFNA, provided they met risk criteria and had not been pregnant or lactating within the prior 12 months. Risk criteria included any one of the following: first- or second-degree relative diagnosed with breast cancer under the age of 60; prior breast biopsy showing proliferative breast disease, atypical hyperplasia, lobular carcinoma *in situ*, multiple biopsies regardless of histology, visual mammographic density 50% or higher; atypia by RPFNA; BRCA1/2 mutation carrier; 5-year BCRAT (available at https://bcrisktool.cancer.gov) or a 10-year Tyrer-Cuzick IBIS version 5 (available at http://www.ems-trials.org/riskevaluator) model risk of at least two times the population risk for age group as listed in the model. Mammogram was not required for women not yet undergoing routine screening but women who were undergoing yearly screening must have had a normal mammogram within 9 months of RPFNA.

RPFNA was to be scheduled between day 1 and 10 of the menstrual cycle in women having at least four menstrual periods in the past year. If not menstruating because of prior endometrial ablation, intrauterine device, or prior hysterectomy, timing of follicular phase was predicted by hormone levels (estradiol, progesterone) assessed prior to RPFNA. Initially women on oral contraceptives were excluded but the protocol was later amended to allow their entry. Women using a hormonal type of contraception must have been using the same type for at least 2 months prior to RPFNA.

Tissue eligibility required hyperplasia, with or without atypia, plus Ki-67 of ≥2% assessed on a minimum of 500 epithelial cells. The lower limit for Ki-67 was based on a prior report which suggested that Ki-67 staining of 2% or higher in hyperplastic foci was associated with increased risk for development of breast cancer (34). Entry onto the intervention protocol was required within 3 months of the RPFNA along with normal renal, hepatic, and hematologic function. Subjects were also asked not to take antibiotics or flaxseed supplements for 6 weeks prior to baseline sampling for blood lignan levels and entry into the study as well as at re-aspiration at 12 months.

Eligible women were accrued at the University of Kansas Medical Center, Northwestern University (SAK), Dana Farber Cancer Institute (JEG), University of Oklahoma Health Sciences Center (WCD), and Ohio State University (LDY). Initial additional sites, which did not accrue an eligible subject were Duke University Medical Center, University of Arkansas for Medical Sciences, and Fred Hutchinson Cancer Research Center. Protocols for benign tissue sampling by RPFNA (HSC 4601; NCT00291096) and for the study intervention (HSC12377; NCT01276704) were approved by the University of Kansas Medical Center Human Subjects Committee. Separate consents were utilized for the screening and interventional protocols at KUMC and generally at other sites.

Risk calculation

The predicted 10-year risk of developing breast cancer was calculated using the Tyrer-Cuzick IBIS version 5 algorithm (available at http://www.ems-trials.org/riskevaluator). Calculations were performed centrally at the University of Kansas based on data collected at the accrual sites.

Specimen acquisition and processing for biomarker assessments

Blood (fasting) and tissue (nonfasting) for biomarker assessments were obtained at baseline and 12 months. Sera and plasma were stored in aliquots at −80°C after processing. RPFNA was performed day 1 to 10 (follicular phase) of the menstrual cycle to reduce Ki-67 variability and minimize bleeding. Two sites per breast were aspirated under local anesthesia with five passes per breast in the outer quadrant and four per breast inner quadrant presuming minimal bleeding or ∼20 passes total per subject via four anesthetized sites (33). The needle tip was preferentially guided to areas of increased resistance. The first two aspiration passes per site (8 of the 20 total passes per subject) were pooled in a 2-mL cryovial containing 0.25 mL PBS, immediately immersed in liquid nitrogen and transferred to a −80°C freezer within 12 hours for later central use in gene-expression assays. The remaining material was pooled in a single 15 cc tube with 9 mL of CytoLyt and 1 mL of 10% formalin. Frozen blood and tissue specimen were batch shipped to the University of Kansas on dry ice for later use and the CytoLyt and formalin-fixed specimens were transferred on blue cold packs via express mail for immediate use.

Within 72 hours of aspiration, CytoLyt fixed specimens were pelleted, washed in CytoLyt, and transferred to PreservCyt. Aliquots were then transferred to slides via ThinPrep methodology for pap staining for cytomorphology or Ki-67 using an RNase-free technique.

Cytomorphology

A categorical estimate of the number of ductal epithelial cells present was made as 500 to 1,000, 1,000 to 5,000, or >5,000. Cytomorphology was assessed by a single cytopathologist (CMZ) and classified by both a categorical method as non-proliferative, hyperplasia, borderline hyperplasia with atypia, or hyperplasia with atypia, and by a Masood semiquantitative index score (35, 36). Index scores of 11 to 14 generally correlate with hyperplasia without atypia, 15 to 18 with hyperplasia with atypia, and 19 to 24 as suspicious for malignancy (36). Cytomorphologic assessments were made without knowledge of the results of the Ki-67 assessment.

Ki-67

Antigen retrieval was performed with 10 mmol/L citrate buffer, pH 6, in a BioCare decloaking chamber (Walnut Creek) for 2 minutes at 120°C. Slides were then stained with MIB-1 mAb (M7240; Dako Cytomation) at a 1:20 dilution in a Dako

Autostainer. Slide(s) with >500 epithelial cells were evaluated for Ki-67. Cell clusters containing the highest proportion of cells staining for Ki-67 were preferentially evaluated. The number of cells with unequivocal nuclear staining out of 500 cells assessed was recorded for each of two independent readers (TM and TP). In case of a difference between the two readers, the scores were averaged.

Gene expression by RT-qPCR

Total RNA was extracted from frozen RPFNA samples using TRizol LS (Life Technologies) according to the manufacturer's instructions, with additional RNA purification using an RNeasy MinElute Cleanup Kit (Qiagen). The RNA collected was thus reflective of adipocytes, stroma, and epithelial cells. RNA was amplified using MessageAmpII aRNA Amplification Kit (Life Technologies) and reverse-transcribed to cDNA using SMARTScribe Reverse Transcriptase (Clontech Laboratories, Inc.) and random nonamer primers. qPCR was performed for selected estrogen response genes, *BRCA1*, and selected genes associated with an inflammatory response. Baseline and post-intervention specimens were assessed together. PCR reactions were run on an Applied Biosystems Prism 7000 Sequence Detection System or a Roche LightCycler 96. Tested transcripts included *ESR1* for ERα, *ESR2* for ERβ, trefoil factor 1 (*TFF1*) for pS2, growth regulation by estrogen in breast cancer 1 (*GREB1*), progesterone receptor (*PGR*), amphiregulin (*AREG*), *BRCA1, IL10*, and *CCL2* for MCP-1. The cycle threshold mean value for each transcript (from duplicate assays per specimen) was normalized using two reference transcripts (*PPIA*, peptidylprolyl isomerase A, for cyclophilin A and *CDKN1B* for cyclin-dependent kinase inhibitor 1B) that showed the least change over time, and a breast tissue standard. Relative levels of each transcript were calculated using the ΔΔCt method.

Cluster analysis

To discern molecular subgroups based on the expression signature of genes of interest, we performed an unsupervised clustering analysis using gene expression measurements collected at baseline and 12 months. The objective of this analysis was to identify subgroups of participants on the basis of their expression. Unsupervised clustering analysis was performed by fitting a Gaussian-distributed Recursively Partitioned Mixture Model (37) to the log2-transformed fold change (logFC) values calculated for each gene using the function glcTree in the R package RPMM. Fold change (FC) values were computed for each gene by dividing its 12-month expression value by the baseline expression value. With the exception of the maxlevel argument, the maximum depth to recurse, which was set to 2 due to the modest number of participants with expression profiling at baseline and 12 months ($n = 77$), all other parameters in the glcTree function were set to their default values. Heat maps were created to visualize the clustering results.

To understand the potential clinical/biological relevance of the identified clusters, we conducted a series of analyses aimed at examining the relationship between cluster membership and select clinical variables collected on participants, including participant age at baseline, change in BMI (12 months baseline), change in estradiol, change in progesterone, change in bioavailable estradiol, change in SHBG, change in Masood score, age at menarche, and parity. ANOVA models and nonparametric Kruskal–Wallis tests were used to examine the association between cluster membership and continuous variables, whereas a Chi-square test and Fisher exact test were used to examine the association between cluster membership and categorical variables.

Hormones

Fasting blood was stored frozen at −80°C until assay. Each subject's pre- and posttreatment samples were run together in duplicate on the same 96-well plate, along with a pooled sera control, plus the kit's standards and controls. Fasting blood was obtained for assays of estradiol, progesterone, and sex hormone binding globulin (SHBG). Commercial kits from Diagnostics Biochem Canada (Dorchester ONT, Canada) were used for enzyme immunoassay of estradiol (CAN-E-430), progesterone (CAN-P-305), and ELISA of SHBG (CAN-SHBG-4010). Limit of detection for estradiol was 10 pg/mL with 8% to 10% intra- and interassay coefficient of variation. Limit of detection for progesterone was 0.1 ng/mL with 10% coefficient of variation. Bioavailable estradiol was calculated according to standard formulae (38).

Other biospecimens collected for secondary or modifying biomarkers

Additional biospecimens were collected for potential later use including fasting serum for adipocytokines and cytokines, buccal swabs for enzyme polymorphisms responsible for lignan metabolism, and overnight urine for urinary lignan measurement.

Adverse events and patient-reported outcomes

Phone contacts were made by the study coordinator at 1, 3, and 9 months to elicit adverse events (AEs) and answer questions. Queries for potential AEs were also performed at the 6- and 12-month in person visits at the time of pick-up of new study agent supply and repeat RPFNA, respectively.

In addition to AEs, participants were asked to complete a Breast Cancer Prevention Trial (BCPT) Symptom Checklist (39) and a breast pain questionnaire at baseline and 12 months (40), as well as keep a menstrual diary.

Statistical analysis

The accrual goal of 242 subjects enrolled was intended to provide a minimum of 220 evaluable subjects with at least baseline and 12-month RPFNA specimens assessed for Ki-67 (the primary endpoint). This was projected to provide 83% power to detect an absolute 2.5% reduction in Ki-67 for the treatment group compared with no reduction in the control group based on an assumption of a common SD of 6% for change in Ki-67 in both the no oral contraceptive and oral contraceptive groups and a type I error rate of 5%.

Table 1. Baseline characteristics for 177 women who were randomized and received study agent.

Characteristics	Placebo $N = 62$	SDG $N = 115$	P
Continuous variables	Median (range) Mean ± SD		Mann–Whitney test
10-Year IBIS risk (%)	4.3 (0.3–23.5) 4.9 ± 3.9	3.9 (0.5–23.1) 5.3 ± 5.0	0.76
Age at baseline RPFNA (years)	40 (24–49) 39.7 ± 6.1	39 (22–49) 38.4 ± 6.7	0.23
BMI (kg/m^2)	24.9 (13.3–38.8) 25.8 ± 5.4	24.2 (17.0–38.5) 25.2 ± 5.4	0.33
Age at menarche (years)	13 (10–17) 12.8 ± 1.4	13 (9–18) 12.9 ± 1.5	0.51
Age at first live birth (years)	28 (17–37) 27.2 ± 5.7	28 (16–40) 27.6 ± 4.8	0.95
Categorical variables	Number		Fisher exact
Current use of oral contraceptives	(percent of randomization arm)		test
No	55 (89%)	108 (92%)	0.58
Yes	7 (11%)	9 (8%)	
Parity			
Nulliparous	15 (25%)	39 (35%)	0.17
Parous	45 (74%)	73 (65%)	
Prior biopsy			
No	34 56%)	70 (63%)	0.42
Yes	27 (44%)	42 (38%)	
Prior history of atypical hyperplasia			
No	57 (92%)	106 (92%)	1.00
Yes	5 (8%)	9 (8%)	
Number relatives with breast cancer			
0	18 (29%)	26 (23%)	0.73
1	40 (66%)	79 (71%)	
2	3 (5%)	5 (5%)	
3	0 (0%)	1 (1%)	

Although the variable for the primary endpoint (change in Ki-67 over time) was normally distributed, this was not the case for most continuous variables. This, plus the limited sample sizes, necessitated the use of nonparametric statistical approaches throughout. For assessment of change in continuous values over the course of the intervention, the Wilcoxon signed-rank test was used. For comparison between groups (e.g., randomization arms), the Mann–Whitney or Kruskal–Wallis tests were used. For categorical variables, Fisher exact tests were used. A P value of <0.05 was the criterion for a statistically significant difference. No correction for multiple comparisons was made given that many secondary analyses were being conducted. Thus, results should be interpreted with caution.

Results

Accrual, retention, and compliance

A total of 434 women were screened for the study by RPFNA (307 from KUMC; 127 from other sites). A total of 254 women screened by RPFNA did not meet all eligibility criteria, primarily because Ki-67 was <2%. A total of 180 women met all criteria, signed a consent to participate in the intervention portion of the trial, and were randomized (115 at KUMC and 65 from other sites). Three women did not receive any study agent; the remaining 177 (115 SDG, 62 placebo) constitute the analytic cohort for this report (**Table 1**; see Supplementary Fig. S1 for Consort Diagram).

A total of 152 women completed the trial with an off-study RPFNA and were evaluable for the primary endpoint (101 from KUMC and 51 from other sites). A total of 11 of the 25 with premature discontinuation were randomized to placebo (18% of randomized) and 14 (11% of randomized) to SDG. Medical- or symptom-related reasons for early discontinuation were new neoplasm in two (DCIS, desmoid; both in placebo), unplanned pregnancy in four (two each from SDG and placebo), oily skin or acne in two (one each SDG and placebo), GI symptoms in one (SDG), and menstrual abnormalities in one (placebo). Hormonal alterations after randomization by patient choice accounted for three (stop or start hormonal contraceptives or oophorectomy—all on SDG arm). Of the remaining 12, six withdrew consent or were noncompliant (three each SDG and Placebo) and six moved out of area or were otherwise lost to follow-up (four SDG, two placebo). Eighty-nine percent of primary endpoint evaluable subjects were from KUMC and Northwestern. The first subject was

accrued at KUMC in January 2011 and the last individual completed study on May 31, 2017.

Compliance was measured by pill count. Median compliance was 96% for the SDG group and 90% for the placebo group, computed for the 140 women who returned study agent at 12 months.

Baseline calculated risk and clinical risk variables

Median 10-year Tyrer-Cuzick (IBIS) risk at baseline was 4% with no differences between SDG and placebo between estimated risk and contributing variables (**Table 1**). Median age was 39, median BMI was 24 kg/m^2, 30% were nulliparous, 8% had a prior biopsy with atypical hyperplasia, and 75% had at least one relative with breast cancer.

Baseline and change in risk biomarkers

Estrogen, SHBG, and progesterone

There was no significant difference between baseline, 12-month or change over time in serum levels of sex hormone binding globulin, estrogen, or bioavailable estrogen for the 149 women who completed the trial and for whom initial and 12-month hormone levels were available (**Table 2**). There was a borderline significant decrease in progesterone levels for the placebo group ($P = 0.083$, Wilcoxon).

Benign breast tissue Ki-67 and cytomorphology

A total of 152 women (86% of those who were randomized and received any study agent) had an RPFNA performed at 12 months and were thus evaluable for the primary endpoint.

Median baseline Ki-67 was 4.1% with no significant difference between the placebo and SDG arms (**Table 3**). The median Masood score was 14 in each group. Fifty-three percent of the 152 women evaluable for the primary endpoint had hyperplasia with atypia at baseline. Comparison of within and between group change in cytomorphology between baseline and 12 months is given in **Table 3**. There was a significant reduction in Masood cytology score both for women randomized to placebo or to SDG ($P = 0.005$ and 0.003, respectively, Wilcoxon) but no difference between the randomization arms. There was no significant difference over time in the proportion of cases given the categorical descriptor of hyperplasia with atypia.

A significant reduction of Ki-67 between baseline and 12 months was observed for women randomized to SDG with a median absolute change of −1.8% and relative change of −44% ($P = 0.001$, Wilcoxon). However, 12-month Ki-67 was also reduced in women randomized to placebo, with a median absolute change of −1.2% and relative change of −40% ($P = 0.034$, Wilcoxon; **Table 3**). Proliferation measured

Table 2. Baseline and 12-month values and change over time for serum levels of systemic hormones.

Biomarker, units	Median (mean ± SD)		*P*
	Placebo; *N* = 49	SDG; *N* = 100	Mann–Whitney test
Estradiol (pg/mL)			
Baseline	79 (132 ± 184)	76 (128 ± 205)	0.77
12-month	75 (106 ± 99)	76 (115 ± 158)	0.95
Change over time	1 (−26 ± 121)	−4 (−13 ± 72)	0.99
Relative change (%)	0 (0 ± 37)	−7 (5 ± 46)	0.94
Within group (Wilcoxon test)	0.42	0.24	
Estradiol (pmol/L)			
Baseline	294 (489 ± 682)	281 (475 ± 759)	0.77
12-month	279 (392 ± 365)	280 (426 ± 583)	0.99
Change over time	0 (−96 ± 447)	−16 (−49 ± 266)	0.99
Relative change (%)	0 (0 ± 37)	7 (−5 ± 46)	0.92
Within group (Wilcoxon test)	0.37	0.24	
SHBG (nmol/L)			
Baseline	69 (98 ± 73)	70 (91 ± 65)	0.93
12-month	72 (92 ± 61)	79 (96 ± 73)	0.87
Change over time	−2 (−6 ± 44)	1 (5 ± 49)	0.31
Relative change (%)	−6 (5 ± 54)	1 (9 ± 45)	0.36
Within group (Wilcoxon test)	0.37	0.57	
Bioavailable estradiol (pmol/L)			
Baseline	3.6 (5.9 ± 9.9)	3.2 (5.4 ± 9.2)	0.98
12-month	3.0 (4.7 ± 5.3)	3.3 (5.0 ± 8.9)	0.94
Change over time	0.0 (−1.2 ± 6.5)	−0.1 (−0.4 ± 2.5)	0.82
Relative change (%)	1 (3 ± 41)	−4 (5 ± 50)	0.77
Within group (Wilcoxon test)	0.67	0.33	
Progesterone (ng/mL)			
Baseline	0.38 (1.85 ± 5.16)	0.36 (1.14 ± 2.41)	0.78
12-month	0.37 (1.50 ± 4.36)	0.43 (1.28 ± 2.59)	0.35
Change over time	−0.03 (−0.35 ± 2.47)	0.0 (0.14 ± 1.31)	0.058
Relative change (%)	−8.5 (23.0 ± 111.4)	0.74 (72.5 ± 252.1)	0.19
Within group (Wilcoxon test)	0.083	0.36	

Table 3. Baseline and 12-month values and change over time for Ki-67, Masood score, and cytomorphology.

Variable and time/change	Placebo $N = 51$ Median Mean ± SD		SDG $N = 101$ Median Mean ± SD	*P* (Mann–Whitney test)
Ki-67 (% positively stained cells by IHC)				
Baseline	3.8		4.4	0.34
	5.4 ± 4.3		5.9 ± 4.5	
12-month	2.2		2.6	0.84
	4.0 ± 4.4		4.5 ± 5.5	
Change over time	−1.2		−1.8	0.72
	−1.4 ± 5.3		−1.4 ± 6.0	
Relative change (percent)	−40		−44	0.70
	5 ± 130		−7 ± 109	
Within group (Wilcoxon test)	0.034		0.001	
Masood score				
Baseline	15		15	0.90
	14.5 ± 1.0		14.5 ± 0.9	
12-month	14		14	0.57
	13.7 ± 1.9		13.9 ± 1.6	
Change over time	−1		0	0.44
	−0.8 ± 1.8		−0.6 ± 1.8	
Within group (Wilcoxon test)	0.005		0.003	
Categorical descriptor		Frequencies		χ^2
Baseline				
Hyperplasia	9 (18%)		17 (17%)	0.92
Borderline atypia	16 (31%)		29 (29%)	
Atypia	26 (52%)		55 (55%)	
12 months nonproliferative	5 (10%)		9 (9%)	0.70
Hyperplasia	13 (26%)		24 (24%)	
Borderline atypia	11 (22%)		22 (22%)	
Atypia	22 (43%)		46 (46%)	
Within group (Wilcoxon test)	0.23		0.33	

by Ki-67 varies substantially between the follicular and luteal phases of the menstrual cycle largely due to high luteal phase progesterone acting in concert with estradiol (41). We measured serum progesterone and identified 35 women with levels indicative of discordant phases of the menstrual cycle at baseline and off-study. Change in Ki-67 was then assessed in a *post hoc* analysis for the remaining 117 participants (42 placebo, 75 SDG), who by progesterone levels appeared to be in the same phase of the cycle at both sampling times. The median change in Ki-67 in the placebo arm for women whose menstrual cycle phase appeared concordant on and off study was nonsignificant at −1.0%; $P = 0.14$, Wilcoxon) but continued to be highly significant at −2.2% for women randomized to SDG ($P = 0.002$, Wilcoxon; **Fig. 2B** and **D**). There was no significant difference in change in benign breast Ki-67 over time between the SDG and placebo groups ($P = 0.72$, Mann–Whitney; **Fig. 2A** and **C**).

Breast tissue gene expression

RT-qPCR was performed on 77 pairs of minimally bloody archived RPFNA specimens for *ESR*1, *ESR2, TFF1, GREB1, AREG, PGR, ADIPOQ, LEP, IL10, CCL2*, and BRCA1. Twenty-two pairs exhibited robust changes (defined as <0.5 or >2.0) in *ESR1* gene expression. with 7 of 10 increases in the placebo arm and 10 of 12 decreases in the SDG arm ($P = 0.018$, Wilcoxon). This resulted in a statistically significant difference between the two arms ($P = 0.015$, Mann–Whitney).

Unsupervised cluster analysis of individual gene expression changes was performed on the entire cohort of 77 and identified four clusters into which all subjects could be grouped (**Fig. 3A**). A similar analysis was conducted on five specific subsets of subjects defined by randomization arm and by the criterion of women being in the same phase of the menstrual cycle at baseline and off-study tissue acquisition. The identified clusters for the entire cohort as well as the subsets were subsequently examined for association with clinical variables, including change in Ki-67, bioavailable estradiol, and progesterone, as well as several known risk factors. For the entire cohort ($N = 77$), there was a statistically significant difference between the four clusters for change in estradiol and bioavailable estradiol ($P = 0.007$, Kruskal–Wallis). Specifically, Cluster 2 (characterized predominately by decreases in early estrogen response genes) was associated with greater decreases in estradiol levels (**Fig. 3B**). There were additional suggestive but not statistically significant associations between clusters and clinical variables. A complete listing of these for the six sets of subjects analyzed, as well as heatmaps of the clusters derived for the six datasets are provided in the Supplementary File.

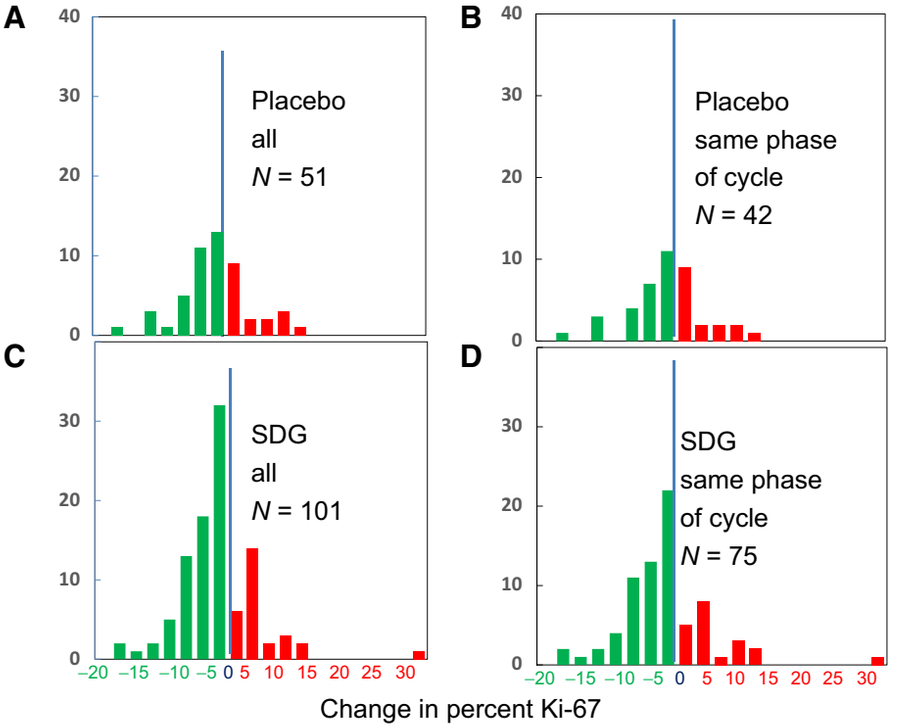

Figure 2.
Comparison of the distribution of change in Ki-67 over time for all evaluable subjects (**A** and **C**) and for those in the same phase of the cycle by progesterone levels at baseline and 12 months (**B** and **D**). Subjects in panels **C** and **D** received SDG; those in **A** and **B** were assigned to placebo.

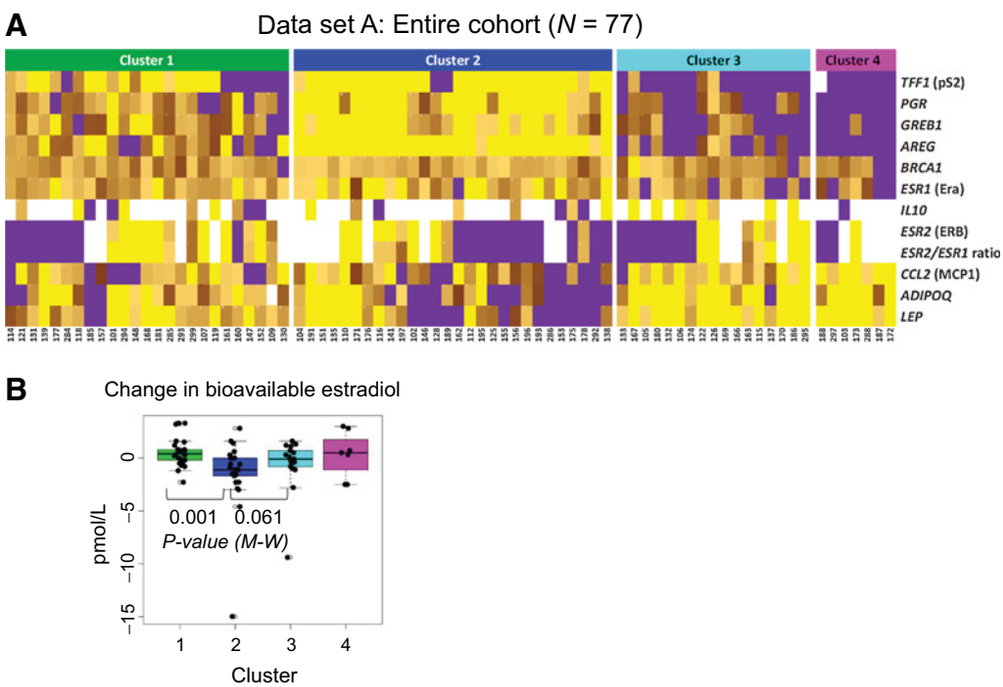

Figure 3.
Representation of the results of analysis of modulation of gene expression. **A,** Heat map demonstrating the results of unsupervised clustering analysis of RT-qPCR data. For individual subjects, an increase in gene expression (fold-change) >2 is indicated by purple and a decrease in gene expression <0.5 is indicated by yellow, with intermediate values indicated by the variable shades of brown. The 77 subjects with paired specimens are separated into four clusters, primarily on the basis of alterations in the estrogen early response genes *TFF1, PGR, GREB1, AREG, ESR1*, and *ESR2*. **B,** Box plot demonstrating the difference in change in bioavailable estradiol levels between the four clusters identified for the entire cohort. Additional details are available in the Supplementary File.

Adverse events

There was no difference in the incidence of adverse events between women randomized to placebo and SDG. Twenty-seven percent of women in each arm reported no adverse events. Thirty-one percent of women on SDG versus 21% of those on placebo reported any grade 1 event, 34% on SDG versus 39% on placebo reported any grade 2 event, and 8% on SDG versus 13% on placebo reported any grade 3 event. There was a total of six subjects for whom a serious adverse event (SAE) was reported; four on the placebo arm (6.5%) and two on the SDG arm (1.7%). The four placebo-arm SAEs included (i) left vestibular disorder leading to a diagnosis of vestibular schwannoma (grade 2); (ii) musculoskeletal connective tissue disorder leading to a diagnosis of desmoid tumor (grade 3); (iii) sinus disorder leading to septoplasty for a deviated septum (grade 3); and (iv) diagnosis of DCIS (grade 3). The two SAEs on the SDG arm involved (i) grade 3 nausea, aseptic meningitis, dehydration, and gastroenteritis; and (ii) unintended pregnancy that resulted in a miscarriage requiring a D&C procedure (grade 5 for fetal death). The worst grade AE reported per subject in each randomization arms in provided in the Supplementary File.

Patient-reported outcomes

The primary patient-reported outcomes were the BCPT symptoms scale and a breast pain score. The BCPT score has seven scales or domains for symptoms experienced over the prior 4 weeks, with intensity rated between 0 and 4 for hot flashes, nausea and vomiting, bladder control, musculoskeletal pain, cognitive problems, and weight problems. Women randomized to placebo had a marginal but statistically significant worsening of symptoms over 12 months (median values for the average of all seven scales increased from 0.03 to 0.06; $P = 0.024$, Wilcoxon). Women randomized to SDG had a stable symptom score (0.04 at baseline and 0.03 at 12 months). This resulted in a statistically significant difference between the randomization groups for the change in average symptom scale values ($P = 0.036$, Mann–Whitney). Median baseline pain score intensity was 1 with no evidence of difference in change of pain intensity between groups.

Discussion

Reduction in breast tumor Ki-67 after short-term treatment is used as a surrogate biomarker predictive of favorable disease-free survival in neoadjuvant endocrine therapy trials (42, 43). Although the optimum analytic approach for comparing change in Ki-67 between groups has been debated over the years, relative change is currently utilized (43–45).

As proliferation in hyperplasia is a risk factor for later development of breast cancer (34), change in Ki-67 in benign breast tissue has also been used in early-phase primary prevention trials as an indicator of likely efficacy and a signal to progress to later phase cancer incidence trials (46). The recent demonstration of reduced breast cancer incidence with low dose tamoxifen in women with atypical hyperplasia and *in situ* cancer (47) was predicted by the much earlier demonstration of decreased Ki-67 in fibroadenomas after ~2 months of a low-dose regimen (48).

Although change in Ki-67 in the SDG arm of our randomized trial was significant ($P = 0.0014$) and very similar to our pilot, the borderline significant reduction of Ki-67 in the placebo arm ($P = 0.034$) resulted in no difference between arms and a null primary endpoint for the randomized trial as designed ($P = 0.72$ for difference). In the short-term window of opportunity trial reported by Thompson and colleagues (49), a daily muffin containing 0 or 25 g of flaxseed (0 or 50 mg SDG) was given to postmenopausal women with newly diagnosed breast cancer for an average of 4 weeks. Tumor Ki-67 labeling index showed a significant median relative decrease of 34% in the flaxseed arm. However, similar to our study, the control group exhibited a 20% relative reduction, such that there was no significant difference between the two groups.

There are multiple potential reasons for reduction in Ki-67 in the placebo arm including inability to ensure baseline and follow-up aspirations in the same phase of the menstrual cycle, lower hormone levels with early menopause transition in some cases, as well as sampling, technical and scoring variance (41, 50). Indeed, when we looked only at change in Ki-67 in women who could be verified by progesterone levels to be in the same phase of the menstrual cycle at baseline and re-aspiration, we continued to find a significant change in Ki-67 in the SDG arm ($P = 0.002$) but not in the placebo arm ($P = 0.14$). There was no statistically significant difference between the two groups for change in Ki-67. Whether significance would have been achieved had there been more subjects is open to speculation.

Given the problems with variability of proliferation in benign breast tissue in premenopausal women by cycle phase, should we even use Ki-67 as a primary surrogate indicator of response in this setting? Perhaps not unless we have a potent treatment such as tamoxifen which is likely to result in a two-third or greater relative reduction in proliferation, a more reliable but convenient method of checking for menstrual cycle phase, or ability to accrue several thousand individuals to a trial. Nonetheless, the lack of any increase in Ki-67 relative to placebo suggests that at worst a moderate dose of flaxseed or SDG in supplements is unlikely to be associated with increased risk for breast cancer. This is important as flaxseed and/or SDG is often used as a dietary supplement to improve insulin sensitivity, promote cardiovascular health, and reduce inflammation, breast pain, and vasomotor symptoms (2, 3, 8). Indeed, women randomized to SDG had a marginally reduced symptom score using the seven domain BCPT scale with no change in the placebo group.

Our limited gene expression studies in benign breast tissue focused on *ESR1* (ERα), *ESR2* (ERβ), and those genes downstream of ERα activation. There was some evidence of decrease in *ESR1* mRNA but as a group no consistent change in *ESR2, BRCA1, AREG, GREB1*, or *pS2* for women randomized to SDG despite animal data suggesting

modulation with lignans (10, 51–53). Individual change in estrogen early response genes appeared related more to individual change in systemic hormones.

Our companion preclinical study of SDG in preventing development of carcinogen-induced mammary cancer in the ABI rat model using doses of SDG producing enterolactone levels similar to our pilot trial found reduction in dysplasia and Ki-67 at 3 months but little change in *Esr1, Esr2*, or *Pgr* mRNA (54).

Recent research suggests that much of the effect of lignans may be as a result of anti-inflammatory influences, which we have not as yet adequately explored in our clinical trial specimens (55–57). Indeed, our companion study in an immunocompetent mouse model of triple negative breast cancer suggested that SDG, in doses producing equivalent enterolactone and enterodiol blood levels as in our human pilot study, suppressed NFκB activation (14). This was associated with growth suppression of triple negative breast cancer with high initial levels of NFκB. A decrease in crown-like structures was also noted in non-tumor-bearing mammary tissue. Among the many genes in the NFκB pathway that were downregulated in the animal study (e.g., *Ccl2, Bcl2a1a, Birc3, Egr2, Mmp9, Tnf*), we assessed *CCL2* which encodes for MCP-1, which is downstream of NFκB activation and associated with breast tumorigenesis (17, 18). We also examined *IL-10*, which is involved in stress response, including interference with NFκB activation (19). Although little change was detected for *CCL2* or *IL-10*, the baseline mRNA levels were low.

Reserved blood, benign breast tissue, and urine specimens from our clinical trial will permit further investigation into systemic mammalian lignan levels, urine estrogen metabolites, and change in breast tissue NFκB activation (phosphorylated p65) as well as downstream gene expression, which in turn may explain individual tissue proliferation and ER-targeted gene expression.

Conclusions

Twelve months of the lignan SDG significantly reduced proliferation in benign breast tissue of premenopausal women at increased risk for development of breast cancer; however, with the unanticipated reduction in proliferation in the placebo arm, there was no significant differences between the two groups. SDG in a dose of 50 mg/day is well tolerated and there is no evidence of adverse effects from this supplement.

Disclosure of Potential Conflicts of Interest

C.J. Fabian reports receiving grants from Komen for the Cure, nonfinancial support from Lignan Research Inc., and grants from Breast Cancer Research Foundation during the conduct of the study. W.C. Dooley reports receiving other support from Shaga Medical LLC outside the submitted work; in addition, W.C. Dooley has a patent to Breast Microedoscope for biopsy pending. J.R. Klemp reports receiving grants and personal fees from Pfizer, personal fees from AstraZeneca, and personal fees from Novartis outside the submitted work. C. Jernigan reports receiving personal fees from University of Kansas Cancer Center outside the submitted work. B.F. Kimler reports grants from Susan G. Komen, grants from Breast Cancer Research Foundation, and nonfinancial support from Lignan Research, Inc., during the conduct of the study. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: C.J. Fabian, J.R. Klemp, C. Jernigan, B.K. Petroff, S.D. Hursting, B.F. Kimler

Development of methodology: C.J. Fabian, T.A. Phillips, B.K. Petroff, B.F. Kimler

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.J. Fabian, S.A. Khan, J.E. Garber, W.C. Dooley, L.D. Yee, J.R. Klemp, J.L. Nydegger, K.R. Powers, A.L. Kreutzjans, C.M. Zalles, T. Metheny, T.A. Phillips, B.K. Petroff

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.A. Phillips, J. Hu, D.C. Koestler, P. Chalise, N.K. Yellapu, B.F. Kimler

Writing, review, and/or revision of the manuscript: C.J. Fabian, S.A. Khan, J.E. Garber, W.C. Dooley, L.D. Yee, J.R. Klemp, J. Hu, D.C. Koestler, C. Jernigan, B.K. Petroff, S.D. Hursting, B.F. Kimler

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.L. Nydegger, T.A. Phillips, B.F. Kimler

Study supervision: C.J. Fabian

Other (studied cytology specimens and rendered evaluation of the contents): C.M. Zalles

Acknowledgments

The authors thank Lignan Research, Inc., which provided the study agent. This study was supported in part by a Susan G. Komen Promise Grant (KG101039) partially funded by Zumba Fitness, LLC, a Susan G. Komen Research Grant (SAC110051), and grants from the Breast Cancer Research Foundation (BCRF-17-049 and BCRF-18-049). This project utilized the KUMC Biostatistics & Informatics Shared Resource, which was supported by NCI Cancer Center Support Grant P30 CA168524.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 31, 2020; revised March 11, 2020; accepted April 15, 2020; published first April 20, 2020.

References

1. Reddy L, Odhav B, Bhoola KD. Natural products for cancer prevention: a global perspective. J Pharmacol Ther 2003;99:1–13.
2. Adolphe JL, Whiting SJ, Juurlink BH, Thorpe LU, Alcorn J. Health effects with consumption of the flax lignan secoisolariciresinol diglucoside. Br J Nutr 2010;103:929–38.
3. Adlercreutz H. Lignans and human health. Crit Rev Clin Lab Sci 2007; 44:483–525.
4. Touré A, Xu X. Flaxseed lignans: source, biosynthesis, metabolism, antioxidant activity, bioactive components, and health benefits. Compr Rev Food Sci Food Saf 2010;9:261–9.
5. Fukumitsu S, Aida K, Ueno N, Takahashi Y, Kobori M. Flaxseed lignan attenuates high-fat diet-induced fat accumulation and induces adiponectin expression in mice. Br J Nutr 2008;100: 669–76.

6. Mani UV, Mani I, Biswas M, Kumar SN. An open-label study on the effect of flax seed powder (Linum usitatissimum) supplementation in the management of diabetes mellitus. J Diet Suppl 2011;8:257–65.
7. Hutchins AM, Brown BD, Cunnane SC, Domitrovich SG, Adams ER, Bobowiec CE. Daily flaxseed consumption improves glycemic control in obese men and women with pre-diabetes: a randomized study. Nutr Res 2013;33:367–75.
8. Prasad K. Flaxseed and cardiovascular health. J Pharmacol Ther 2009; 54:369–77.
9. Peterson J, Dwyer J, Adlercreutz H, Scalbert A, Jacques P, McCullough ML. Dietary lignans: physiology and potential for cardiovascular disease risk reduction. Nutr Rev 2010;68:571–603.
10. Saggar JK, Chen J, Corey P, Thompson LU. The effect of secoisolariciresinol diglucoside and flaxseed oil, alone and in combination, on MCF-7 tumor growth and signaling pathways. Nutr Cancer 2010;62: 533–42.
11. Truan JS, Chen JM, Thompson LU. Comparative effects of sesame seed lignan and flaxseed lignan in reducing the growth of human breast tumors (MCF-7) at high levels of circulating estrogen in athymic mice. Nutr Cancer 2012;64:65–71.
12. Morad V, Abrahamsson A, Kjölhede P, Dabrosin C. Adipokines and vascular endothelial growth factor in normal human breast tissue in vivo - correlations and attenuation by dietary flaxseed. J Mammary Gland Biol Neoplasia 2016;21:69–76.
13. Velalopoulou A, Tyagi S, Pietrofesa RA, Arguiri E, Christofidou-Solomidou M. The flaxseed-derived lignan phenolic secoisolariciresinol diglucoside (SDG) protects non-malignant lung cells from radiation damage. Int J Mol Sci 2015;17:pii: E7.
14. Bowers LW, Lineberger CG, Ford NA, Rossi EL, Punjala A, Camp KK, et al. The flaxseed lignan secoisolariciresinol diglucoside decreases local inflammation, suppresses NFκB signaling, and inhibits mammary tumor growth. Breast Cancer Res Treat 2019; 173:545–57.
15. Khongthong P, Roseweir AK, Edwards J. The NF-κB pathway and endocrine therapy resistance in breast cancer. Endocr Relat Cancer 2019;26:R369–80.
16. Toriola AT, Appleton CM, Zong X, Luo J, Weilbaecher K, Tamimi RM, et al. Circulating receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL), and mammographic density in premenopausal women. Cancer Prev Res 2018;11:789–96.
17. Katanov C, Lerrer S, Liubomirski Y, Leider-Trejo L, Meshel T, Bar J, et al. Regulation of the inflammatory profile of stromal cells in human breast cancer: prominent roles for TNF-α and the NF-κB pathway. Stem Cell Res Ther 2015;6:87.
18. Wang W, Nag SA, Zhang R. Targeting the NF-κB signaling pathways for breast cancer prevention and therapy. Curr Med Chem 2015;22: 264–89.
19. Vasto S, Candore G, Balistreri CR, Caruso M, Colonna-Romano G, Grimaldi MP, et al. Inflammatory networks in ageing, age-related diseases and longevity. Mech Ageing Dev 2007;128:83–91.
20. Velentzis LS, Cantwell MM, Cardwell C, Keshtgar MR, Leathem AJ, Woodside JV. Lignans and breast cancer risk in pre- and postmenopausal women: meta-analyses of observational studies. Br J Cancer 2009;100:1492–8.
21. Horn-Ross PL, John EM, Lee M, Stewart SL, Koo J, Sakoda LC, et al. Phytoestrogen consumption and breast cancer risk in a multiethnic population—the Bay Area Breast Cancer Study. Am J Epidemiol 2001; 154:434–41.
22. Zamora-Ros R, Ferrari P, González CA, Tjønneland A, Olsen A, Bredsdorff L, et al. Dietary flavonoid and lignan intake and breast cancer risk according to menopause and hormone receptor status in the European Prospective Investigation into Cancer and Nutrition (EPIC) Study. Breast Cancer Res Treat 2013;139:163–76.
23. Linseisen J, Piller R, Hermann S, Chang-Claude J. Dietary phytoestrogen intake and premenopausal breast cancer risk in a German case–control study. Int J Cancer 2004;110:284–90.
24. Cotterchio M, Boucher BA, Kreiger N, Mills CA, Thompson LU. Dietary phytoestrogen intake—lignans and isoflavones—and breast cancer risk (Canada). Cancer Causes Control 2008;19:259–72.
25. Lowcock EC, Cotterchio M, Boucher BA. Consumption of flaxseed, a rich source of lignans, is associated with reduced breast cancer risk. Cancer Causes Control 2013;24:813–6.
26. Touillaud MS, Thiébaut AC, Fournier A, Niravong M, Boutron-Ruault MC, Clavel-Chapelon F. Dietary lignan intake and postmenopausal breast cancer risk by estrogen and progesterone receptor status. J Natl Cancer Inst 2007;99:475–86.
27. Tetens I, Turrini A, Tapanainen H, Christensen T, Lampe JW, Fagt S, et al. Dietary intake and main sources of plant lignans in five European countries. Food Nutr Res 2013;57:1.
28. Sonestedt E, Wirfält E. Enterolactone and breast cancer: methodological issues may contribute to conflicting results in observational studies. Nutr Res 2010;30:667–77.
29. Kilkkinen A, Pietinen P, Klaukka T, Virtamo J, Korhonen P, Adlercreutz H. Use of oral antimicrobials decreases serum enterolactone concentration. Am J Epidemiol 2002;155:472–7.
30. McCann SE, Wactawski-Wende J, Kufel K, Olson J, Ovando B, Kadlubar SN, et al. Changes in 2-hydroxyestrone and 16alpha-hydroxyestrone metabolism with flaxseed consumption: modification by COMT and CYP1B1 genotype. Cancer Epidemiol Biomarkers Prev 2007;16:256–62.
31. 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.
32. Zeleniuch-Jacquotte A, Adlercreutz H, Shore RE, Koenig KL, Kato I, Arslan AA, et al. Circulating enterolactone and risk of breast cancer: a prospective study in New York. Br J Cancer 2004; 91:99–105.
33. Fabian CJ, Kimler BF, Zalles CM, Klemp JR, Petroff BK, Khan QJ, et al. Reduction in Ki-67 in benign breast tissue of high-risk women with the lignan secoisolariciresinol diglycoside. Cancer Prev Res 2010;3:1342–50.
34. Shaaban AM, Sloane JP, West CR, Foster CS. Breast cancer risk in usual ductal hyperplasia is defined by estrogen receptor-alpha and Ki-67 expression. Am J Pathol 2002;160:597–604.
35. Zalles C, Kimler BF, Kamel S, McKittrick R, Fabian CJ. Cytologic patterns in random aspirates from women at high and low risk for breast cancer. Breast J 1995;1:343–9.
36. 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.
37. Houseman EA, Christensen BC, Yeh RF, Marsit CJ, Karagas MR, Wrensch M, et al. Model-based clustering of DNA methylation array data: a recursive-partitioning algorithm for high-dimensional data arising as a mixture of beta distributions. BMC Bioinformatics 2008;9:365.
38. Endogenous Hormones and Breast Cancer Collaborative Group. Free estradiol and breast cancer risk in postmenopausal women: comparison of measured and calculated values. Cancer Epidemiol Biomarkers Prev 2003;12:1457–61.
39. Stanton AL, Bernaards CA, Ganz PA. The BCPT symptom scales: a measure of physical symptoms for women diagnosed with or at risk for breast cancer. J Natl Cancer Inst 2005;97:448–56.
40. Khan SA, Apkarian AV. The characteristics of cyclical and non-cyclical mastalgia: a prospective study using a modified McGill Pain Questionnaire. Breast Cancer Res Treat 2002;75:147–57.

41. Navarrete MA, Maier CM, Falzoni R, Quadros LG, Lima GR, Baracat EC, et al. Assessment of the proliferative, apoptotic and cellular renovation indices of the human mammary epithelium during the follicular and luteal phases of the menstrual cycle. Breast Cancer Res 2005;7:R306–13.
42. Ellis MJ, Tao Y, Luo J, A'Hern R, Evans DB, Bhatnagar AS, et al. Outcome prediction for estrogen receptor-positive breast cancer based on postneoadjuvant endocrine therapy tumor characteristics. J Natl Cancer Inst 2008;100:1380–8.
43. Klintman M, Dowsett M. Early surrogate markers of treatment activity: where are we now? J Natl Cancer Inst Monogr 2015;51:24–8.
44. Dowsett M, Smith IE, Ebbs SR, Dixon JM, Skene A, Griffith C, et al. Short-term changes in Ki-67 during neoadjuvant treatment of primary breast cancer with anastrozole or tamoxifen alone or combined correlate with recurrence-free survival. Clin Cancer Res 2005;11: 951s–8s.
45. Johnston S, Puhalla S, Wheatley D, Ring A, Barry P, Holcombe C, et al. Randomized phase II study evaluating palbociclib in addition to letrozole as neoadjuvant therapy in estrogen receptor-positive early breast cancer: PALLET trial. J Clin Oncol 2019;37:178–89.
46. Fabian CJ, Kimler BF, Mayo MS, Khan SA. Breast-tissue sampling for risk assessment and prevention. Endocr Relat Cancer 2005;12: 185–213.
47. DeCensi A, Puntoni M, Guerrieri-Gonzaga A, Caviglia S, Avino F, Cortesi L, et al. Randomized placebo controlled trial of low-dose tamoxifen to prevent local and contralateral recurrence in breast intraepithelial neoplasia. J Clin Oncol 2019;37:1629–37.
48. de Lima GR, Facina G, Shida JY, Chein MB, Tanaka P, Dardes RC, et al. Effects of low dose tamoxifen on normal breast tissue from premenopausal women. Eur J Cancer 2003;39:891–8.
49. Thompson LU, Chen JM, Li T, Strasser-Weippl K, Goss PE. Dietary flaxseeds alters tumor biological markers in postmenopausal breast cancer. Clin Cancer Res 2005;11:3828–35.
50. Olsson H, Jernström H, Alm P, Kreipe H, Ingvar C, Jönsson PE, et al. Proliferation of the breast epithelium in relation to menstrual cycle phase, hormonal use, and reproductive factors. Breast Cancer Res Treat 1996;40:187–96.
51. Chen J, Saggar JK, Corey P, Thompson LU. Flaxseed and pure secoisolariciresinol diglucoside, but not flaxseed hull, reduce human breast tumor growth (MCF-7) in athymic mice. J Nutr 2009;139:2061–6.
52. Mueller SO, Simon S, Chae K, Metzler M, Korach KS. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ERα) and ERβ in human cells. Toxicol Sci 2004;80:14–25.
53. Vissac-Sabatier C, Coxam V, Déchelotte P, Piherit C, Horcajada MN, Davicco MJ, et al. Phytoestrogen-rich diets modulate expression of Brca1 and Brca2 tumor suppressor genes in mammary glands of female Wistar rats. Cancer Res 2003;63:6607–12.
54. Delman DM, Fabian CJ, Kimler BF, Yeh H, Petroff BK. Effects of flaxseed lignan secoisolariciresinol diglucoside on preneoplastic biomarkers of cancer progression in a model of simultaneous breast and ovarian cancer development. Nutr Cancer 2015;67: 857–64.
55. Abrahamsson A, Morad V, Saarinen NM, Dabrosin C. Estradiol, tamoxifen, and flaxseed alter IL-1β and IL-1Ra levels in normal human breast tissue *in vivo*. J Clin Endocrinol Metab 2012;97: E2044–54.
56. Jaskulski S, Jung AY, Behrens S, Johnson T, Kaaks R, Thone K, et al. Circulating enterolactone concentrations and prognosis of postmenopausal breast cancer: assessment of mediation by inflammatory markers. Int J Cancer 2018;143:2698–708.
57. Lampe JW, Kim E, Levy L, Davidson LA, Goldsby JS, Miles FL, et al. Colonic mucosal and exfoliome transcriptomic profiling and fecal microbiome response to a flaxseed lignan extract intervention in humans. Am J Clin Nutr 2019;110:377–90.