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PROTOCOL FACE SHEET

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Protocol Title: Flaxseed Lignan as a Prevention Strategy for Pre-Menopausal Women at High Risk for Development of Breast Cancer

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Agent(s)/Supplier:
SDG (as Brevail®)/matched placebo)
Lignan Research, Inc.
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List of abbreviations

ADH	Atypical ductal hyperplasia
AE	Adverse event
ALT	alanine aminotransferase
AMH	Antimullerian hormone
AST	aspartate aminotransferase
BCPT	Breast Cancer Prevention Trial
b.i.d.	bis in diem/twice a day
BMD	Bone mineral density
CI	Confidence interval
BMI	Body mass index
BPQ	Breast pain questionnaire
CBC	Complete blood count
CRF	Case Report/Record Form
DCIS	Ductal carcinoma in situ
ECG	Electrocardiogram
EDL	Enterodiol
ENL	Enterolactone
ER	Estrogen receptor
FDA	Food and Drug Administration
FNA	Fine needle aspiration
FSH	Follicle stimulating hormone
IGF1	Insulin-like growth factor 1
IGFBP3	Insulin-like growth factor binding protein 3
IND	Investigational new drug
i.v.	intravenous(ly)
IRB	Institutional review board
IUD	Intrauterine device
KUCC	University of Kansas Cancer Center
KUMC	University of Kansas Medical Center
LCIS	Lobular carcinoma in situ
LH	Lutenizing hormone
p.o.	per os/by mouth/orally
RPFNA	Random periareolar fine needle aspiration
SAE	Serious adverse event
SDG	Secoisolariciresinol diglycoside
SECO	Secoisolariciresinol
SHBG	Sex hormone binding globulin
SOP	Standard operating procedure

SCHEMA

Initial Screening

Premenopausal women with BMI < 40 kg/m^2, age 21-49 who have any of the following risk factors suggesting moderate to increased risk of breast cancer: first or second degree relative diagnosed with breast cancer under the age of 60; prior breast biopsy showing proliferative breast disease, ADH, DCIS, LCIS; multiple biopsies regardless of histology, mammographic density 50% or higher; atypia by RPFNA; BRCA1/2 mutation carrier; 5 yr Gail risk of ≥1.67% or 2X risk for age as given in model, or 10 yr Tyrer-Cuzick risk 2x population risk as listed in model.

No pregnancy or lactation for previous 12 months. If using a hormonal type of contraception, should have been using the same type of hormonal contraception for at least 2 months prior to RPFNA. .

RPFNA must be able to be scheduled between day 1 and 10 of the menstrual cycle in women having at least 4 menstrual periods in the past year.

If not menstruating because of prior endometrial ablation or use of progestin only IUD or prior hysterectomy with ovaries preserved, must be able to predict timing of follicular phase by hormone levels (estradiol, progesterone, FSH) assessed prior to RPFNA.

If undergoing yearly mammographic screening must have normal mammogram within nine months of RPFNA. If not undergoing routine screening, no mammogram is necessary.

↓

Screening Visit for Eligibility

No flaxseed supplements or antibiotics for the 3 weeks prior to RPFNA.

Random breast FNA performed during follicular phase (Day 1-10) of menstrual cycle or if non-menstruating additional serum aliquot frozen for confirmation of follicular phase.

Serum and plasma specimens frozen for later assays in central laboratories.

CBC and **fasting** metabolic chemistry profile may be performed if desired and study entry anticipated within 3 months of RPFNA

History and physical exam may be performed if desired and study entry anticipated within 3 months of RPFNA

Subject given materials for menstrual diary recording

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Tissue Qualifiers for trial participation

Epithelial hyperplasia +/- atypia; Masood score ≥13; plus Ki-67 ≥ 2%

↓

Study Entry Visit *(within six months of RPFNA)*

History and physical exam (if not performed within 3 months)

Complete BCPT symptom checklist, BPQ Breast Pain Questionnaire and Lignan Diet Questionnaire. Collect Pre-study Menstrual Diary Information

CBC and **fasting** metabolic chemistry profile (if not performed within previous 3 months)

Fasting serum and plasma specimens frozen for later assays in central laboratories.

Spot (+ optional overnight) urine collection and buccal sample for later assays in central laboratories.

Negative urine pregnancy test

Randomization number assigned (next in sequence)

Study Agent dispensed – 6 month supply

↓

Phone contacts at 1 and 3 months to monitor adverse events and concomitant meds

↓

6-month Visit
Review of adverse events and concomitant meds
Return study agent for compliance check; New supply of study agent issued

↓

Phone contact at 9 months to monitor adverse events and concomitant meds

↓

2-4 Weeks Prior to Anticipated Off Study Visit (for non-menstruating women only)
Assess serum hormone levels (Estradiol, Progesterone, FSH); use for prediction of menstrual cycle and scheduling of RPFNA during follicular phase.

↓

12-month Visit (Off-Study)
No antibiotics for the 3 weeks prior to RPFNA.
Fasting blood drawn, preferably within 3-4 hours of last dose of study agent
CBC and metabolic chemistry profile
Serum and plasma specimens frozen for later assays at central laboratories.
Spot (+ optional overnight) urine collection frozen for assay in central laboratories.
Complete BCPT symptom checklist, BPQ Breast Pain Questionnaire, Lignan Diet Questionnaire,
Collect menstrual Diary Information.
Return Study Agent.
RPFNA (Day 1-10 of menstrual cycle); for non-menstruating women, additional serum aliquots frozen for confirmation of follicular phase.

↓

Phone contact at 2 weeks off study agent to monitor adverse events

1.0 OBJECTIVES

The overall objective is to determine whether the natural flaxseed lignan, secoisolariciresinol diglycoside (SDG), in amounts providing plasma lignan levels associated with reduced breast cancer risk, will favorably modulate risk biomarkers for breast cancer development. This will provide biologic evidence of SDG's potential for breast cancer chemoprevention. Based on a positive pilot study in which both Ki-67 and the proportion of women with atypical cytomorphology was reduced with 12 months of SDG 50 mg/day, a randomized, placebo-controlled clinical chemoprevention trial will be conducted wherein pre-menopausal women at increased risk for the development of breast cancer will receive Brevail® (200 mg, equivalent to 50 mg of SDG) or matched, blinded placebo for 12 months. A total of 242 subjects will be accrued, randomized 2:1 to SDG vs placebo, with the goal of achieving 220 subjects completing study and evaluable for biomarker change.

The primary objective is to determine if women randomized to 12 months of SDG exhibit greater change in the proportion of breast epithelial cells expressing the proliferation marker Ki-67/MIB-1 in hyperplastic benign breast tissue than do women randomized to placebo.

Secondary objectives are:

1) to determine if 12 months of SDG is associated with favorable modulation of other risk biomarkers including cytologic atypia, and serum levels of bioavailable estradiol and testosterone, IGF-I:IGFBP-3 ratio, c-peptide, adiponectin, and leptin.
2) to determine if Ki-67 and modulation of other risk biomarkers is correlated with change in blood or urine lignans.
3) to determine impact or potential impact on quality of life as measured by the BPQ breast pain questionnaire (a modified McGill Pain questionnaire), The Breast Cancer Prevention Trial (BCPT) Symptom Checklist a menstrual diary for cyclicity and cycle length for women not on cycle modulating contraceptives`; and antimullerian hormone (AMH), a measure of follicular reserve.

An exploratory objective is to interrogate SDG's mechanism of action through measurement of changes in hormones, growth factors, gene expression, adipokines and cytokines and proteomic signatures**.**

The overriding hypothesis is that biomarkers assessed both in serum and in hyperplastic breast tissue obtained by RPFNA from high risk pre-menopausal women will be favorably modulated by 12 months of SDG without negatively affecting quality of life.

Specifically, it is hypothesized that SDG administration will be associated with

- a reduction in proliferation as measured by Ki-67;
- a reduction in proportion of specimens with cytologic evidence of atypia;
- a favorable tolerability and quality of life profile;
- no significant impact on menstrual cycle length;
- no significant effect on follicular reserves as measured by early follicular phase anti-mullerian hormone in women entering the study between ages 21-35;
- a reduction in breast pain in those women entering the study with mastalgia.

2.0 BACKGROUND

2.1 Need for Prevention Alternatives to Tamoxifen in Premenopausal Women

It is currently estimated that of the ~194,000 invasive breast cancers anticipated in the US this year, 24% or ~ 40,000 will be in women under 50, and most women <50 will be premenopausal [1]. A high proportion of women developing breast cancer have no major risk factors and the future in the US of screening mammography for average risk women under 50 is uncertain [2,3]. Tamoxifen is the only FDA approved drug for risk reduction in premenopausal women considered at high risk because of a prior pre-cancerous biopsy or Gail model estimated 5 year probability of cancer 1.67% or greater [4]. This equates to the average risk of a 60 year old but is about 3X that of the average 40 year old and 10X that of the average 30 year old [5,6]. Although an overview of placebo-controlled trials suggests tamoxifen reduces risk of breast cancer by 38%, no survival advantage has yet been demonstrated for primary prevention [7,8]. Further, uptake by even very high risk women is poor primarily due to concerns about side effects [9-12]. An economical prevention alternative is needed which does not negatively alter quality of life and is so safe that it does not require a prescription or medical monitoring. Such an alternative is likely to be accepted by a larger proportion of women and thus has the potential for greater impact on cancer incidence. Natural products such as lignans with few side effects, which are inexpensive and may be purchased without a prescription, are likely to have high uptake if reduced cancer incidence or favorable modulation of pathways leading to cancer can be demonstrated.

2.2 Reduction in Breast Epithelial Cell Proliferation and Cancer with Lignans

Lignans are a group of phenolic compounds found in edible plants which after fermentation by gut bacteria are converted to derivatives which modulate estrogen dependent and independent signaling pathways [13-15]. The typical Western diet is low in lignans, providing 10 mg or less per day [16,17]. An important plant lignan is Secoisolariciresinol (SECO) found in highest concentration in flaxseed but also in other oil rich seeds, nuts, whole grains, legumes, and certain fruits, and vegetables [16, 17]. The primary medicinal use of lignans in young women is to treat cyclic breast pain [18] and in Canada, flaxseed is considered first line treatment for this condition [19]. Flaxseed or SECO administered as the diglycoside (SDG) is associated with reduced mammary gland cell proliferation and incidence of estrogen receptor negative (ER-) and ER+ mammary cancer in pre-clinical studies [20-23]. SECO is converted to enterolactone (ENL) and enterodiol (EDL) which is readily measured in blood and urine. There is some inconsistency [24-38] in human correlative studies which is to be expected given the inherent limitations of dietary recall, early use of frequency questionnaires with incomplete validation for lignans, variation in lignan metabolism, and single point blood collections [39-43]. However, many studies suggest reduced cancer incidence in premenopausal women with higher lignan intakes or plasma ENL levels [25-28,30-32,36]. These correlative studies indicate reduction in risk of ER- as well as ER+ cancer, including ER- cancer in pre-menopausal women [29,31,33-35]. This is important as ER- tumors constitute approximately 40% of cancers in premenopausal women and at present there is no clinically available agent which has been demonstrated to reduce ER- breast cancer although several are undergoing initial testing [44]. Correlations between lignan exposure and breast cancer incidence appear stronger in premenopausal women with 1 or more CYP 17 A2 alleles which in turn results in higher endogenous estrogen production [25,26,30,36]. Lignans appear to reduce proliferation in established tumors. In a pre-surgical model trial, pre and postmenopausal women with newly diagnosed breast cancer randomized to ~30 days of muffins baked with 25 grams of flaxseed were found to have a significant *reduction* in their tumor proliferation rate (Ki-67) at re-excision lumpectomy or mastectomy compared to Ki-67 change in women eating muffins without flaxseed [45].

2.3 Mechanisms by Which Lignans Block Breast Carcinogenesis

Pre-clinical investigations suggest lignans have diverse effects which may be important in initiation, promotion and progression including

- antioxidant properties [46];
- enhanced differentiation [47] and increased expression of the tumor suppressor proteins BRCA 1 and 2 [48];
- reduction in tissue estrogen production and estrogen receptor related signaling in part due to suppression of inflammatory cytokines and aromatase [49-53];
- reduction in growth factor receptor related signaling and proliferation [51,54,55]; and
- favorable modulation of adiponectin [56,57], insulin [58,59], and leptin [56,60].

These latter risk biomarkers for breast cancer [57,61-66] are important modulators of MAP kinase, PI3 kinase and mTOR pathways [67-75] which link extracellular signals to cellular machinery controlling proliferation and migration, protein synthesis, energy metabolism, and cell survival [76-79] and are thought to play an important role in breast cancer promotion and progression.

2.4 Lignan Pilot Trial

2.4.1 Selection of lignan source and dose for pilot trial

An important early step in evaluating a new intervention for preventive activity is to assess its effects on risk biomarkers for development of cancer, generally over a few months to a year. Tissue risk biomarkers are generally selected as the primary endpoint. If the agent has previously been tested in humans and a dose is reasonably well established which is likely to modulate markers but unlikely to cause symptoms, a single arm pilot feasibility study is first performed to show consistent modulation of markers and feasibility. This is then followed by a Phase II placebo-controlled trial [80]. In preclinical studies, either flaxseed or the lignan SDG appeared to be equally efficacious in reducing breast cancer [20-22] and in the placebo-controlled clinical trial reported by Thompson *et al*., 25 grams of flaxseed was able to reduce proliferation in cancer cells [45]. A pilot study was conducted at KUMC utilizing an SDG dose which would produce plasma ENL levels in the range that had been associated with significant reduction in risk for breast cancer in clinical correlative studies [24] and would be comparable to the 25 grams of flaxseed baked in muffins in the pre-surgical model study showing reduced proliferation in cancer cells [45]. The variability of SDG content and bioavailability in raw flaxseed and the propensity for raw flaxseed to cause gastrointestinal symptoms at higher doses [81] prompted the selection of a commercial preparation (Brevail®) with a consistent SDG content but with reduced likelihood of gastrointestinal symptoms. A single 200 mg Brevail® capsule contains 50 mg of highly bioavailable SDG providing plasma ENL levels similar to that in 25 grams of whole flaxseed. Daily dosing with this formulation produced median ENL levels of 63 nmol/L similar to those in the highest quintile of the case control study of Pietinen *et al*. which was associated with a 2/3 reduction in cancer incidence when compared to ENL levels in the lowest quintile [24,82].

2.4.2 Selection of biomarkers for pilot trial

Given the likelihood that lignans serve as partial estrogen antagonists in a high estrogen environment [52,53,55,83], risk biomarkers were selected that were likely to be modulated by selective estrogen receptor modulators (SERMs), as well as those likely to be modulated by lignans for the pilot trial in premenopausal women [84]. The initial risk biomarker set included cytomorphology and Ki-67 in hyperplastic breast epithelium in tissue sampled by RPFNA,

mammographic breast density, serum bioavailable estradiol, testosterone, and IGF-I:IGFBP-3 [85-90]. Subsequently serum adiponectin, leptin and cytokine assays [57,61-66,91,92] were added given the emergence of data that several of these markers - particularly adiponectin and possibly leptin - may be risk factors for breast cancer. Adiponectin counteracts the promotional effects of leptin on the PI3 kinase and mTOR pathways [93], reduces insulin and insulin resistance [94], and blocks cell entry into S phase [95], whereas cytokines promote tissue aromatase activity, increasing estradiol. Change in Ki-67 in areas of cytologic hyperplasia was chosen as the primary endpoint using the following rationale. It had previously been established in a prospective study that cytologic atypia in tissue sampled by random periareolar fine needle aspiration (RPFNA) was a strong risk biomarker for breast cancer [85]. When utilized in women at increased risk for cancer between the ages of 30 and 60, it was associated with a 5-fold increase in the risk of DCIS and invasive cancer and could stratify short term risk based on the Gail model despite being used in women already at increased risk for the disease [96]. However, hyperplasia with atypia is not a practical primary response endpoint for Phase II prevention studies because based on prior studies [97] it would require screening aspiration of 4-5 pre-menopausal women to enroll one woman with atypia. Further requiring baseline atypia would limit generalizability of results. Consequently, the proliferation biomarker Ki-67 assessed in areas of hyperplasia was chosen as the primary endpoint. Ki-67 is currently acceptable as a primary endpoint in Phase II prevention trials for the following reasons [80]:

- Proliferation plays a fundamental role in carcinogenesis.
- Ki-67 expression is positively associated with pre-cancerous changes of histologic and cytologic atypia [86].
- Higher Ki-67 in hyperplastic and atypical hyperplastic specimens predicts cancer development [87,88].
- Ki-67 can be modulated quickly in both cancer treatment and prevention trials [98-100].

2.4.3 Eligibility criteria for SDG pilot study

Eligibility criteria included regular menstrual cycles, no oral contraceptives, a 3-fold or greater increase in 5-year estimated risk for breast cancer relative to SEER statistics for age group, and normal mammogram performed in the follicular phase of the menstrual cycle within 3 months of baseline RPFNA. Subjects on oral contraceptives were excluded in order to reduce subject hormonal heterogeneity and because it might be more difficult to demonstrate biomarker modulation from SDG in the face of exogenous hormones. Baseline RPFNA was also to be performed in the follicular phase and had to show unequivocal hyperplasia +/- atypia as well as a minimum Ki-67 expression of 2% in hyperplastic ducts. The Ki-67 value of 2% was chosen since this is the median Ki-67 value in hyperplastic tissue obtained by RPFNA from high risk pre-menopausal women in the follicular phase of the cycle [86]. Further, a Mayo Clinic series suggests that women having a biopsy showing atypical hyperplasia and a Ki-67 of 2% or higher have a significantly higher 10-year risk of breast cancer (14% versus 3%) [88]. The early-mid follicular phase is utilized due to greater subject comfort and less bleeding during RPFNA.

2.4.4 SDG pilot study results

Forty-nine premenopausal women were accrued over 2.3 years to the single institution; NCI R21-funded pilot study of 12 months of SDG. Forty-five subjects completed the study and were evaluable for biomarker change. Median age was 43 years, BMI 22.8 kg/m^{2,} and 5 year Gail risk 1.6% in biomarker evaluable subjects. Seventy-five percent had one or more first degree relatives with breast cancer, and 22% had a prior biopsy with atypical ductal hyperplasia or lobular carcinoma in situ.

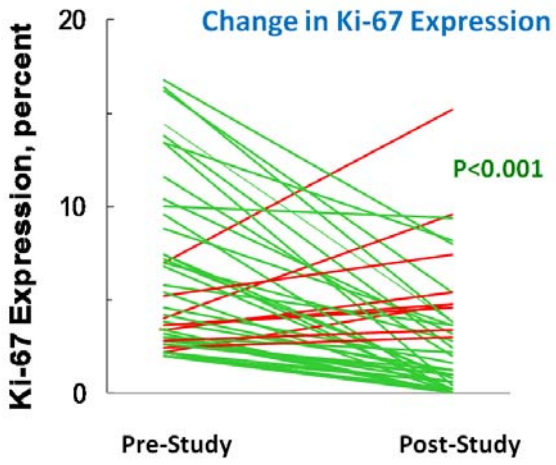
2.4.4.1 Change in lignan levels

Compliance was excellent (median = 96%) with few serious adverse events (4% grade 3). Median plasma ENL increased ~ 9-fold (11 nmol/L to 99 nmol/L). Even greater increases were observed in SECO and EDL, often not detectable at baseline with median levels of 5.7 and 23.3 nmol/L, respectively, at 12 months.

Plasma Lignan	N	Pre-Study, nmol/L	Mid-Study (6 mo), nmol/L	Post-Study nM (12 mo), nmol/L	Difference Baseline to 12 mo, nmol/L
Secoisolariciresinol	42	0.0 0.61 ± 1.16	4.4 32.8 ± 73.7	5.7 33.7 ± 66.5	5.5 32.8 ± 66.5
Enterodiol	42	0.0 0.69 ± 1.29	34.1 55.2 ±62.5	23.3 84.3 ± 133.0	23.3 83.7 ± 133.0
Enterolactone	42	11.1 15.8 ± 17.4	95.2 108.9 ± 67.0	99.2 132.7 ± 120.3	74.8 117.0 ± 117.7

2.4.4.2. Change in Ki-67 and cytomorphology

Thirty-six (80%) of the 45 evaluable subjects demonstrated a decrease in Ki-67, from a median of 4% (range 2-16.8%) to 2% (range 0-15.2%) ($p<0.001$ by Wilcoxon signed rank test). A decrease from baseline in the proportion of women with atypical cytology from 62% to 42% ($p=0.035$) was also observed.



2.4.4.3 Change in blood adipokines and cytokines, growth factors and hormones

Significant increases in serum adiponectin and reductions in leptin were observed along with borderline changes in IGFBP3 and bioavailable testosterone by ELISA techniques [101]. Additional preliminary data on selected serum adipokines and cytokines performed on reserved serum from 37 women using Luminex Multiplex Cytokine Assays, and Multiplex Endocrine Assay kits by Dr. Hursting at the University of Texas at Austin are promising and mirror ELISA data with increases in adiponectin, a significant reduction in leptin, a significant increase in the adiponectin to leptin ratio, and a numerical decrease in the inflammatory cytokine TNF alpha.

Analyte	Pre-Treatment	Post-Treatment	Pre-Post Treatment	P value
Leptin (ng/ml)	15.7 ± 5.3	12.9 ± 5.2	1.22	0.03
Adiponectin (μg/ml)	23.6 ± 10.8	29.3 ± 11.4	0.82	0.03
Leptin:adiponectin Ratio	0.67 ± 0.2	0.49 ± 0.2	1.37	0.02
TNF-α (pg/ml)	5.2 ± 2.3	4.5 ± 1.9	1.15	0.16

2.4.4.4 Change in mammographic density

There were no significant changes in follicular phase percent mammographic density.

2.4.4.5 Adverse events

The main expected AEs from SDG were diarrhea, flatulence, and irregular menses. GI symptoms, usually grade 1, were encountered in 42%, irregular menses in 26%, and hot flashes in 6% of subjects. Only two grade 3 AEs were reported. One subject was diagnosed with DCIS on the basis of the mammogram performed prior to the 12 month RPFNA. This was not considered related to the study agent. One subject discontinued study after 3 months because of grade 3 pelvic pain that was thought to be possibly related to study agent

Frequency of adverse events by worst reported per subject.

	CTC Grade					
Number	0	1	2	3	4 or 5	Total
Total	7 (14%)	17 (35%)	23 (47%)	2 (4%)	0 (0%)	49

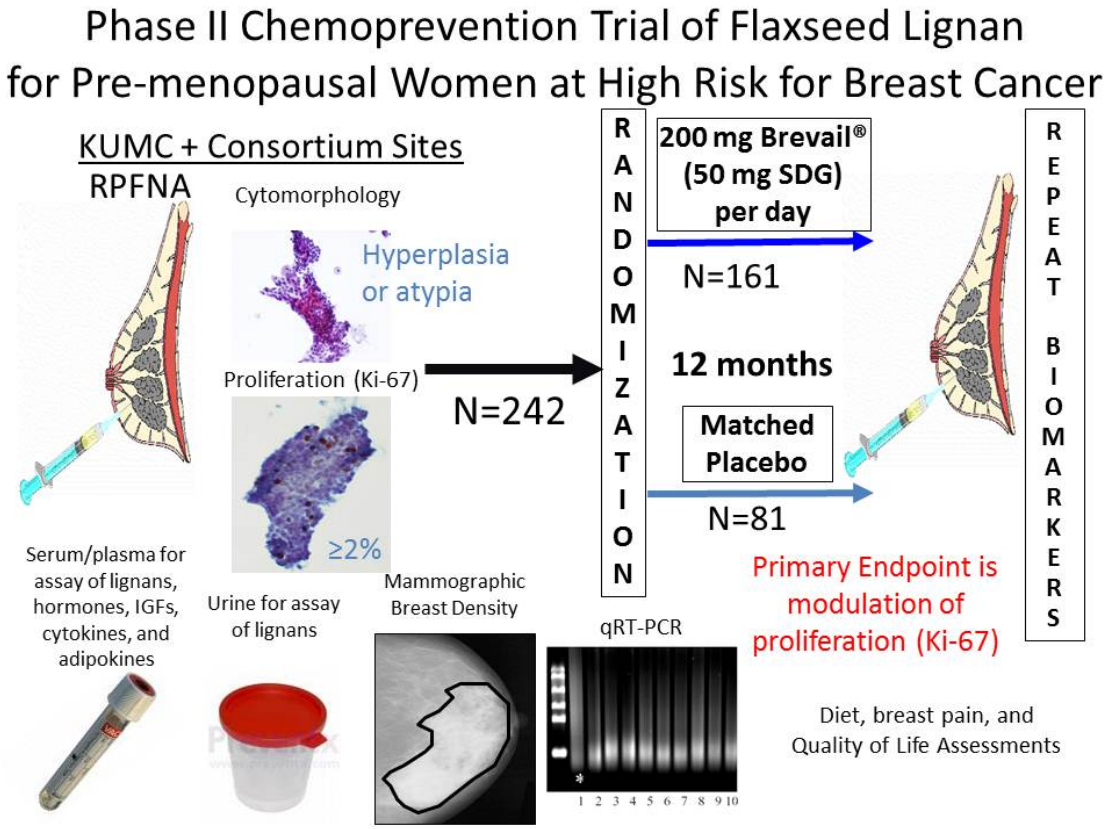
Change in plasma lignans did not correlate with change in Ki-67, adverse events (AEs), or compliance [101].

3.0 SUMMARY OF STUDY PLAN

The favorable outcome of the pilot study suggests progression to a Phase II multi-institutional placebo-controlled trial of 12 months of Brevail® (50 mg SDG) in moderate and high risk premenopausal women. Modulation of Ki-67 in women with baseline Ki-67 of 2% or greater in hyperplastic tissue is the primary endpoint rather than normalization of atypia, so as to limit the number of women who would need to be screened by RPFNA to 2-3 for every woman going on trial (vs. 4-5 if all had to have atypia). Other risk biomarkers will be measured, as well as impact on quality of life, change in breast pain in those with mastalgia at baseline, and surrogate markers of fertility. These considerations will be important for eventual uptake of SDG as a prevention agent. Mechanisms of action will also be explored. Similar eligibility criteria are used as in the pilot study, where the average percentage of cells expressing Ki-67 at baseline was 6.4% (median 4%) with an average change of -3.7% with a common standard deviation of 5.5%.

The study eligibility is being altered approximately 2.5 years after study start to allow inclusion of women on oral contraceptives. This will both widen application of results should they be positive and is expected to at least double the rate of accrual. Assessment of difference in change in Ki-67 between women randomized to SDG versus placebo can be accomplished with a 2:1 randomization of 242 women. Assuming a dropout rate of 10%, with 220 evaluable subjects there will be 83% power to detect a 2.5% reduction in Ki-67 for the treatment group compared to no reduction in the control group; this will be our primary endpoint. This is based on an assumption of a common standard deviation 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%

3.1 Randomized Trial Schema



3.2 Impact and Innovation

The potential impact of this trial is high in that it addresses unmet needs in the area of breast cancer prevention for premenopausal women. It has the potential to provide an intervention which:

1) has excellent tolerability with low likelihood of inducing menopause symptoms, gynecologic problems, or reducing quality of life.
2) is low cost and available without a prescription or need for medical monitoring.
3) is likely to reduce ER- as well as ER+ breast cancers and
4) has potential to reduce ovarian cancer [102].

The combination of lack of objective or subjective side effects, lack of need for prescription or medical monitoring and low cost (Brevail® is less than $20 per month) makes it an intervention that can and is likely to have high proportional uptake and compliance in average as well as high risk women. This contrasts with tamoxifen, the only approved agent for risk reduction in premenopausal women, which still requires a prescription, medical monitoring, and because of side effects is approved only for high risk individuals [4]. Even if the magnitude of SDG related relative risk reduction is only 25% (compared to 38% for tamoxifen), with high uptake 10,000 new cases of breast cancer per year could be prevented in women under age 50. There is no prescription drug likely to provide the uptake of an over-the-counter natural product, and thus even if the relative risk reduction with SDG is less than tamoxifen the impact on cancer incidence with a positive trial is likely to be higher. Given the 2/3 reduction in breast cancer in case control studies for women with high ENL levels (similar to that achieved with 50 mg SDG/day in the pilot study) relative to women with low levels (similar to baseline ENL levels in the pilot), a 25% reduction in risk is a reasonable expectation [24,101].

This will be the only placebo-controlled clinical trial of SDG or flaxseed with sufficient subjects such that a significant difference in the primary endpoint risk biomarker could potentially be observed. Further, it is the only trial taking a comprehensive approach to assessing the molecular mechanisms of action for lignans and their effects on risk biomarkers, quality of life, breast pain, menstrual cycle length and surrogate measures of fertility. The application of state of the art molecular techniques to the exploration of mechanisms of action and critical pathways affected will contribute to acceptance of the trial results by regulatory agencies, peer reviewers, and the lay public.

4.0 PARTICIPANT SELECTION

4.1 Risk Level Required for RPFNA Screening for Eligibility

Given the low probability of side effects and the desire to be able to generalize results to a moderate as well as high risk population, the target cohort is pre-menopausal women who have a relative risk for breast cancer which is 2-fold or greater than that of the average woman in their age group by virtue of **any one of the following conditions**:

4.1.1 A 1st or 2nd degree relative with breast cancer diagnosed under the age of 60;

4.1.2 A prior biopsy indicating proliferative breast disease, atypical hyperplasia, DCIS or LCIS;

4.1.3 Multiple prior breast biopsies regardless of histology;

4.1.4 50% or higher estimated mammographic density on visual inspection;

4.1.5 Prior or current RPFNA evidence of atypia; or

4.1.6 Known carrier of a BRCA1 or 2 mutation.

4.1.7 Projected probability of developing breast cancer by either the Gail model (5-year risk) or the Tyrer–Cuzick model (10-year risk) that is at least 2-fold greater than that for the population. For the Gail model any value of 5-year risk ≥1.67% satisfies the requirement.

4.2 Age, Life-Style and Medical Eligibility Criteria for Tissue Screening

Candidates for tissue screening for this study are pre-menopausal women who meet the risk criteria above and **all** of the following demographic and medical criteria:

4.2.1 Age 21 to 49 (limiting the maximum age to 49 in women who are not already perimenopausal will reduce the possibility of reduction in Ki-67 due to entry into menopause transition during the study).

4.2.2 BMI < 40 kg/m^2.

4.2.3 Stable hormone status for 8 weeks prior to aspiration and willing to maintain same status while on study. This means no change in an oral or non-oral hormonal contraceptive within the past 8 weeks prior to RPFNA. .

4.2.4 Timing of follicular phase of menstrual cycle:

a) For women who are actively menstruating, at least 4 menstrual periods in past 12 months such that there is a reasonable expectation of being able to perform the aspiration in the follicular phase of the cycle (sometime between day 1 and day 10 inclusive, with day one being the first day of bleeding). Very light periods and spotting count.
OR
b) Women who have not had 4 menstrual periods in the past year due to Mirena type IUDs or endometrial ablation may be screened, but hormone levels must be assayed 2-4 weeks prior to RPFNA in order to predict when to perform RPFNA so as to be in

the follicular phase. Lab results and institutional normal ranges must be reviewed by the Protocol Chair, who will provide an acceptable time window within which to conduct the RPFNA. Women who have had their uterus removed, but still have at least one functioning ovary may be screened using the same hormone level check. Must be willing to have same assessment of hormones and prediction of follicular phase repeated for the off-study RPFNA.

4.2.5 If undergoing annual screening mammography, must have been performed within 9 months prior to baseline RPFNA and interpreted either as not suspicious for breast cancer or with any supplementary imaging performed and interpreted as not suspicious for breast cancer.

4.2.6 Breast exam interpreted as normal (not suspicious for cancer).

4.2.7 There has been a sufficient interval between a breast procedure and the RPFNA: at least 2 months for a prior RPFNA, at least 2 months for a core needle biopsy, at least 3 months for an excisional biopsy.

4.3 Exclusion Criteria for Screening RPFNA and Study Participation

Candidates are ineligible for tissue screening if they meet **any** of the following conditions:

4.3.1 Have changed type of hormonal contraception during the previous 8 weeks.

4.3.2 Have experienced lactation or pregnancy during the previous 12 months,

4.3.3 Considered to be perimenopausal and/or entering the menopause transition

4.3.4 Chronic use of systemic antibiotics. Systemic antibiotics reduce intestinal bacteria and thus the ability to convert SECO to ENL. Topical lotions which include antibiotics are permitted. Occasional use of antibiotics is allowed, but must be stopped for 3 weeks prior to RPFNA and for 3 weeks prior to collecting blood or urine specimens for the pre-study (i.e., prior to dispensing/starting study agent) assessment of lignan levels.

4.3.5 Consumption of systemic antibiotics or commercial supplements containing SDG (e.g. flaxseed or sesame seed supplements) during the 3 weeks prior to **baseline RPFNA**. Consumption of foods containing flaxseed or sesame seed are permitted

4.3.6 Use of any selective estrogen receptor modulator or aromatase inhibitor (tamoxifen, raloxifene, arzoxifene, acolbifine, anastrozole, exemestane, letrozole) within the previous 6 months.

4.3.7 Currently enrolled on an interventional investigational study.

4.3.8 Bilateral breast implants.

4.3.9 Invasive cancer diagnosis within five years, excluding squamous or basal cell skin cancer. Subjects with DCIS or stage I invasive cancer are eligible if they are at least 2 months from radiation or surgery and at least 1 yr from chemotherapy or hormone therapy. RPFNA will be performed on the contralateral breast only in these instances.

4.3.10 Any malignancy metastatic to other organ, excluding Hodgkin's or non-Hodgkin's lymphoma.

4.3.11 Regular consumption of non-prescription anticoagulants, such as aspirin, NSAIDS or fish oil during the 3 weeks prior to baseline RPFNA is strongly discouraged, but occasional use will not exclude subject from participation.

4.3.12 Any consumption of prescription anticoagulants, including Coumadin and Lovenox, during the 3 weeks prior to baseline RPFNA.

4.3.13 Any other condition or intercurrent illness that in the opinion of the investigator makes the subject a poor candidate for RPFNA or the trial.

4.4 Inclusion Criteria for Study Entry

4.4.1 RPFNA performed within 6 months of the Study Entry Visit and in the follicular portion (day 1-10) of the menstrual cycle. Note that day 1 is defined as the first day of bleeding. For non-menstruating women, RPFNA during the follicular phase must be confirmed by hormone levels drawn on the day of RPFNA. Either clinical laboratory results are sent to Protocol Chair for assessment of menstrual cycle phase; or an additional frozen serum aliquot is sent to KUMC for assay of hormone levels and phase confirmation. Confirmation of follicular phase will be included in the eligibility report for the potential subject.

4.4.2 RPFNA specimen exhibits hyperplasia +/- atypia; Masood score of $\geq$13 with $\geq$500 cells on the cytology slide.

4.4.3 Ki-67 ≥2% positivity ($\geq$500 cells).

4.4.4 Willing to continue on the same or similar type of contraception during the 12 month course of study. If using non-hormonal contraception, continue that and if hormonal contraception continue that. If heterosexually active, must be agreeable to use some form of contraception during the trial unless husband or partner has had a vasectomy or subject has had uterus removed. Safety of SDG during pregnancy has not been documented.

4.4.5 Have reasonable organ function as documented by CBC and metabolic chemistry profile (within 3 months prior to Study Entry Visit).

4.4.6 Willing to undergo a history and physical at baseline and 12 months and be contacted periodically by the trial coordinator during the 12 month study period.

4.4.7 Willing to repeat RPFNA at 12 months following initiation of study agent.

4.4.8 Willing to have blood drawn at baseline and 12 months. If non-menstruating, an additional draw will be performed at approximately 11 months so as to predict when to perform the RPFNA

4.4.9 Able to understand and willing to provide informed consent for the RPFNA's and study participation.

4.5 Screening, Consent and Enrollment Process

4.5.1 Cohort and Accrual: Potential subjects will be screened in the Breast Cancer Prevention Center at the University of Kansas Medical Center (KUMC), Kansas City, Kansas; and at similar high risk clinics at collaborating institutions. Recruitment will be conducted over approximately 4.5 years for a total accrual of n=242.

4.5.2 Consent at KUMC: At KUMC, potential subjects will sign KUMC HSC#4601 consent for screening RPFNA procedure; subjects qualifying for randomization will sign the trial specific consent document.

4.5.3 Consent at collaborating sites: Collaborating sites may use the trial specific consent prior to screening RPFNA for obtaining biospecimens for baseline assays, or may use a separate approved research/clinical consent for that purpose.

4.5.4 All prospective subjects undergoing RPFNA for screening at collaborating sites will be registered in a site screening log by the site study coordinator or principal investigator. The study coordinator will assign a unique identifier from a pre-designated series specific to that institution. This identifier, which will include subject initials, serves as the subject "name" for screening purposes and along with the date will be attached to all subject related biospecimens sent to KUMC. A screening checklist will also accompany the screening RPFNA specimen. This will also be used for pre-enrollment blood, urine questionnaires, and data collection. No protected health information should be attached to these items.

4.5.5 Subjects may not be stratified and randomized until they have completed the entire eligibility process including RPFNA and collection of baseline biospecimens, and the site study coordinator has received confirmation from KUMC that the subject is tissue eligible, and for non-menstruating women that the RPFNA was performed in the follicular phase.

4.5.6 The RPFNA results provided to the site will include the cytomorphology descriptor (non-proliferative, hyperplasia, or hyperplasia with atypia), cytology index score and Ki-67 percent positive cells; as well as a statement that the subject is or is not eligible on the basis of RPFNA to be randomized and enrolled.

4.5.7 At that time subjects will have an on study visit scheduled at which the remaining clinical eligibility criteria will be confirmed; at least a spot urine (and preferably also a 10-12 hr overnight) urine, fasting blood, and a buccal swab for polymorphism testing will be collected (collection materials provided by KUMC), and baseline questionnaires will be completed.

4.5.8 Once all biospecimens have been collected, all questionnaires completed, and all eligibility criteria have been met, the subject may be randomized and a completed eligibility checklist communicated to the KUMC Project Manager. Subjects will not be considered enrolled on the intervention phase of this study until they have been randomized.

4.5.9 Subjects who have undergone RPFNA tissue screening and are not eligible or who have decided not to participate in the intervention phase prior to randomization will have the primary reason for non-participation recorded on the post-aspiration screening case report form.

4.6 ID Assignment and Randomization

4.6.1 Each site will have a series of unique study IDs that provide the study agent assignment. The site study coordinator will assign the subject the next available sequential study ID. All forms, questionnaires and biospecimens subsequent to randomization are to be labeled with this study ID as well as the date.

4.6.2 Each site investigational pharmacy will have a randomization schedule which has been prepared and provided by the Study Statistician. Study agent randomization to placebo or active SDG will be provided for each sequential post-randomization study ID number. Other than the investigational pharmacist(s) and the central biostatisticians, the enrolling site staff, the coordinating site staff, and subjects will be blinded as to the assignment to active agent or matched placebo.

4.6.3 For subjects not on oral contraceptives, sequential study ID numbers are assigned starting at X001 and then increasing (e.g., X002, X003, etc.). Subjects using oral contraceptives will be assigned a study ID number starting with the last number provided to the institution and then in decreasing order (e.g., X099, X098, X097, etc.).

5.0 AGENT ADMINISTRATION

5.1 Dose Regimen and Dose Groups

Both Brevail® and matched placebo are to be provided by Lignan Research, Inc. to the investigational pharmacy at each participating institution.

Brevail® (containing 50 mg of the active ingredient SDG) or placebo (containing the same filler material but without SDG) will be given to all trial participants for the 12 months of participation. All subjects should have off-study procedures no later than within 36 hours of the last dose of SDG or placebo. However, if a participant stops study agent prematurely in an unscheduled situation, she should come in for biomarker re-assessment as early as possible.

5.2 Study Agent Accountability

It is necessary to maintain a careful record of the inventory and disposition of all study agent using a drug accountability record form. The Investigational Pharmacy at each participating site is required to maintain adequate records of receipt, dispensing and final disposition of study agent. Include on receipt record from whom the agent was received and to whom study agent was shipped, date, quantity and batch or lot number. On dispensing record, note quantities and dates study agent was dispensed to and returned by each participant.

5.3 Packaging and Labels

Brevail® and matched placebo will be supplied by Lignan Research, Inc. in a form ready for dispensing. Each bottle of 100 capsules will be labeled with a label identifying study specific information, such as protocol number, dosing instructions, recommended storage conditions, randomization number, and a caution statement indicating that the agent is limited by United States law to investigational use only and the agent should be kept out of reach of children.

5.4 Storage

Storage in the Investigational Pharmacy should be at room temperature.

5.5 Study Agent Administration

The Institutional Investigational Pharmacy will be responsible for receiving, inventory and dispensing of study agent after documentation of local IRB approval of the KUMC-approved protocol and consent.

Study agent will be dispensed with the study number of each subject. All subjects will be given 200 capsules (two bottles) of study agent at the baseline visit. This provides sufficient study agent to accommodate a 2-3 week delay in scheduling of procedures or travel. If additional study agent is required because of extended delays in scheduling, it may be shipped to the subject. At the 6-month visit, the unused study agent will be counted for compliance, but then returned to the subject. An additional 200 capsules (two bottles) will be dispensed for the remainder of the study. Again, if additional study agent is required because of extended delays in scheduling, it may be shipped to the subject.

The study coordinator will give the study agent to the subject with instructions to take one capsule at approximately the same time of day in conjunction with intake of food and at least eight ounces of water and to keep track of missed doses or lost capsules.

5.6 Blinding and Unblinding Methods

In the event of a serious adverse event or other circumstance requiring unblinding, in the opinion of the responsible physician, the site investigator will notify KUMC. The KUMC statistician will unblind the subject to the site investigator if necessary. If the treatment blind is broken, the reason and the date should be recorded and signed by the investigator.

5.7 Agent Destruction/Disposal

At the completion of investigation, all unused study agent will be destroyed in accordance with each site's investigational pharmacy regulations

5.8 Concomitant Medications

Subjects are not allowed to start flaxseed, or lignan supplements, tamoxifen, raloxifene, anastrozole, letrozole or any other SERM or aromatase inhibitor or investigational drug while participating on this trial. Subjects may not alter their use of oral or non-oral hormonal contraceptives.

Subjects are strongly encouraged to continue any routine medications throughout the duration of the study to avoid confounding adverse events due to starting and stopping other medications while on study.

All medications (prescription and over-the-counter), vitamin and mineral supplements, and/or herbs taken by the participant will be documented on the concomitant medication CRF and will include: 1) start and stop date, dose and route of administration, and indication. Medications taken for a procedure (e.g., biopsy) should also be included.

5.9 Dose Modification

Dose modifications will not be utilized for this study. Because study agent is only available in a single dose capsule, no dose modification option is available. If a subject is experiencing adverse events or is having a medical procedure that requires a brief suspension of SDG, this must be noted in the CRF on the dose suspension page. At the discretion of the PI, subjects may be removed from participation for significant dose suspension time periods.

Subjects with grade 2 adverse events considered to be related to study agent will have the agent suspended until the toxicity has resolved. The agent will then be resumed. If toxicity again occurs, and is thought to be agent-related, the agent will be permanently stopped. Grade 2 hot flashes or menstrual irregularities will not be cause for suspending study agent.

Subjects who experience grade 3-4 toxicity will have the study agent suspended. The agent may be re-instituted after resolution of the adverse event, providing the investigator feels relatively certain that the toxicity is not due to the study agent and that resuming agent will not affect recovery from the condition

5.10 Interruption or Discontinuation of Treatment

A genuine effort must be made to determine the reason(s) why a subject fails to return for the necessary visits or is discontinued from the trial. Information regarding the reason for not completing the trial will be recorded on the appropriate case report forms.

It will be documented whether or not each subject completed the clinical study. If for any subject study treatment or observations were discontinued the reason will be recorded on the appropriate case report form. Reasons that a subject may discontinue participation in a clinical study are considered to constitute one of the following:

- adverse event(s)
- abnormal laboratory value(s)
- abnormal test procedure result(s)
- subject develops breast cancer
- protocol violation
- subject withdrew consent
- lost to follow-up
- administrative problems
- physician decision
- death

All subjects that discontinue trial prematurely require notification of the Protocol Chair or her designee at KUMC within 72 hours of known discontinuation.

Any subject who receives at least one dose of study agent will be included in the safety analysis.

5.11 Adherence/Compliance

At the end of treatment the actual quantity of unused study agent will be compared to the anticipated amount of unused study agent. At the six month visit, after being counted by the study coordinator, the unused agent will be returned to the subject.

Subjects that maintain 70% of study agent intake as measured through patient reports and tablet counts conducted by the trial coordinator will be considered “compliant”. Percent intake will be computed as the number of tablets taken divided by the number of days subject should have taken capsules. Pill counts and compliance will be considered separately for the first six and the last six months in the analysis.

6.0 CLINICAL EVALUATIONS AND PROCEEDURES

6.1 Schedule of Events

Evaluations Procedures	Eligibility/ Pre-study visit	On Study Visit	Phone Contacts Months 1,3,9	6 Month Visit	11 Month Visit	12 Month Visit	2 Week Follow up Phone Contact
Informed Consent	X	X					
Height/Weight, Vital Signs	X	X				X	
Breast Exam	X[c]	X[c]				X	
Serum hormone assay only if non-menstruating	X[d]				X[d]		
RPFNA (day 1-10 of menstrual cycle)	X[a]					X[a]	
Serum/Plasma collection for hormone + lignan assays	X[b]					X[b]	
Screening eligibility checklist	X						
CBC + Fasting blood for chem. Profile, lignan and cytokine c-peptide	X[bc]					X[b]	
Urine collection (spot required, additional overnight preferred, materials to subject for menstrual diary)		X[b]				X[b]	
Medical History	X[c]						
Physical Exam	X[c]					X	
Urine Pregnancy Test		X					
Buccal sample		X[b]					
BCPT Symptom Checklist, BPQ Breast pain and lignan intake questionnaires		X				X	
Concomitant Meds.		X	X	X		X	X
Dispense Study Agent		X		X			
Adverse Events			X	X		X	X
Return Study Agent/Evaluate Compliance				X		X	
Off Study Evaluation							X

[a] See specimen management section 7.0 for details on shipping of RPFNA materials to KUMC

[b] To be shipped to KUMC for central analysis. See specimen management section 7.0. Notify KUMC by fax and email prior to shipment.

[c] Physical, Breast exam, Baseline CBC, Medical history and fasting chem profile may be performed at RPFNA or Study Entry Visit as long as within 3 months prior to study entry.

[d] If non-menstruating, blood assayed for hormone levels 2-4 weeks prior to anticipated RPFNA so as to predict timing of follicular phase.

6.2 Pre-Study Procedures and Evaluations

6.2.1 Mammography and Clinical Breast Exam.

Pre-study mammography is not a requirement for this study unless the potential subject is of sufficient risk for development of breast cancer that they are undergoing annual screening. In that case potential subjects must have had a normal mammogram within 9 months of baseline RPFNA. Subjects must have a clinical breast exam at baseline that is judged as being non-suspicious for breast cancer.

6.2.2 Pre-RPFNA instructions

Use of systemic antibiotics must be stopped for 3 weeks prior to RPFNA. Subjects will be told to discontinue aspirin products, fish oil, vitamin E, and NSAIDs at least 3 weeks prior to the RPFNA to reduce risk of excessive bleeding. Subjects should also be told to take 10 mg of vitamin K for 3 days prior to the RPFNA and to eat a good breakfast the day of the procedure. Vitamin K is strongly advised to reduce bleeding, but failure to take Vitamin K will not render a subject ineligible. Likewise, failure to discontinue fish oil or Vitamin E, or ingestion of an occasional aspirin or NSAIDS, will not render a subject ineligible. If subjects take an anti-anxiety medication such as ativan the day of the procedure they must have someone drive them home from the procedure. Otherwise they should be able to drive themselves.

For non-menstruating women (see section 4.2.4), hormone levels (estradiol, progesterone, and FSH) must be assayed 2-4 weeks prior to RPFNA in order to predict when to perform RPFNA so as to be in the follicular phase. Lab results and institutional normal ranges must be reviewed by the Protocol Chair, who will provide an acceptable time window within which to conduct the RPFNA.

6.2.3 Pre-study Eligibility Visit - RPFNA Procedure

Breast tissue acquisition will be performed between day 1 and 10 of the menstrual cycle. RPFNA will be performed under local anesthesia from both breasts (two sites on each breast or one unaffected breast in the case of prior DCIS or invasive cancer). The breast skin will first be cleansed with betadine and alcohol swabs. Two weals per breast with local anesthesia (typically 1% lidocaine with added epinephrine 1:100,000 buffered with bicarbonate) are made using a tuberculin (1 cc) syringe in the skin at ~ 2 and 10 o'clock within 4 cm of the areola, avoiding obvious blood vessels. After skin weals are placed, 4-5 cc of the same mixture is injected deeper into the breast tissue at the 2 sites. For women who have a hypersensitivity to epinephrine, lidocaine without added epinephrine should be used.

Six needle passes per site for the outer quadrants and four for the inner quadrants are performed with pre-wetted 10-12 cc syringes (sterile RPMI or PBS) with 21 gauge 1½ inch needle, within the anesthetic weal sites to withdraw epithelial cells lining the ductal tree. The clinician will preferentially sample areas in which resistance (density) is appreciated with the tip of the needle.

At KUMC and institutions with liquid nitrogen available in the clinic, the first two passes from each site in the breast will be expelled into labeled cryovials containing PBS (provided by KUMC) and immediately flash frozen by placing in liquid nitrogen. It is critical that bloody material not be placed into these cryovials as it ruins the assays.

Material from the remaining 14 passes (20 passes for institutions not participating in the flash frozen RPFNA substudy) in both breasts will be expelled and pooled into a single labeled 15 cc tube containing modified Cytolyt™ (9 cc Cytolyt™ and 1 cc 10% neutral buffered formalin). Fluid from the Cytolyt™ tube should be drawn into the needle/syringe hub approximately four times when expelling material into tube solution. Cell material should not be expressed against the side of the tube. Tubes are then placed on a tube rocker for approximately 12-24 hours prior to shipment to KUMC.

After the procedure, subjects will have ice packs placed on the breast for 10-15 minutes to control swelling and bleeding. Subjects are then wrapped with gauze cling wrap to create pressure on the puncture sites. A snug bra is also strongly recommended. Subjects are advised to limit strenuous physical activity over the next 24 hours and to report any pain or signs of infection.

Subjects may be offered analgesics after the procedure in order to control any pain that may occur.

6.2.4 Pre-study Blood work performed at that time of the RPFNA visit

Note: blood drawing kits will be provided to sites with tubes and vials, plus processing and storage information. These kits may be updated as needed and new directions for processing will take precedence over instructions listed below.

6.2.4.1 Pre-study serum + plasma collection for assays at central laboratories (non-fasting)

Two 4 ml gold top gel clot tubes will be collected for estradiol, SHBG, testosterone and progesterone. Blood is allowed to clot for 30 minutes at room temperature and is then centrifuged at ~2200 x g for 15 minutes. A minimum of 0.5 to 1 ml of serum is placed in each of at least four cryovials pre-labeled with subject screening identifier, date, and type of specimen (e.g., serum). They are to be frozen at -20°C until can be transported to a -80°C freezer within 2 hours for storage until they can be shipped to KUMC. For non-menstruating women, an additional cryovial containing at least 0.5 ml of serum is sent frozen immediately to KUMC for confirmation of follicular phase. Alternatively, immediate assessment of estradiol, progesterone, and FSH may be conducted at the institutional clinical laboratory and the results sent to the Protocol Chair for interpretation and confirmation of follicular phase.

One lavender top 4 ml EDTA plasma tube for lignans should be drawn and centrifuged at ~400 x g for 15 minutes. Two cryovials with a minimum of 0.5 to 1 ml of plasma should be labeled with Study ID, date, “plasma”. These may be temporarily stored at 4°C but should be moved to -20°C within 1 hour and to -80°C within 2 hours of collection.

The total tubes for this visit are two 4-ml gold top and one 4-ml lavender top; except for non-menstruating women for whom an additional 1-2 two 4-ml gold top tubes are required..

6.2.5 Completion of Risk Information

Subjects should be given a copy of the family history form to complete. This information is used to calculate and IBIS risk profile (central office). This worksheet should be completed and sent back to the central office prior to, or the day of the Study Entry Visit. The completed calculation will then be sent back to the investigator. The risk information CRF should be completed after the site receives the completed calculation.

6.2.6 Menstrual Diary

Menstrual diary materials will be given to women to begin completing prior to on study visit, indicating days of blood flows or spotting.

6.3 On Study Visit/Baseline Studies

6.3.1 Physical and Medical History

Subjects will have a brief medical history and physical (including clinical breast exam) performed by a study-associated clinician. Forms which may be used as source documents for medical history and physical will be provided by KUMC.

6.3.2 Clinical Laboratory Assessments

Blood for CBC and a fasting metabolic chemistry profile may be obtained and assayed at the institution or any CLIA approved lab at the RPFNA visit or at any time within 3 months prior to randomization.

6.3.3 Specimens collected for central laboratory assays
Systemic use of antibiotics must be stopped for 3 weeks prior to collecting blood or urine specimens for the pre-study (i.e., prior to dispensing/starting study agent) assessment of lignan levels.

6.3.3.1 Urine collection

A spot urine sample should be collected during the clinic visit. Approximately three drops should be used to perform a urine pregnancy test and the results should be recorded. Urine lignans are stable at room temperature for up to a week, but site personnel should decant two 12-ml aliquots in labeled 15 ml tubes as soon as possible and store at -80°C until shipment to KUMC. Site personnel should record urine volume as well as the time of collection.

Preferably, a 10-12 hour overnight urine specimen will also be collected and brought to the clinic. Site personnel should decant two 12-ml aliquots in labeled (Study ID, date, “urine”) 15 ml tubes as soon as possible and store at -80°C until shipment to KUMC. Site personnel should record urine volume as well as the beginning and ending times of collection.

6.3.3.2 Fasting blood collection (for on study visit)

Subjects should be fasting for at least 10 hours. The time of last food consumption and the time of collection should be recorded.

6.3.3.2.1 Fasting serum for Adipokines, Cytokines and C-peptide

Two red top 4 ml serum clot tubes should be drawn, allowed to clot for 30 min at room temperature, and then centrifuged at ~3000 x g for 5 minutes (at 4º C, a refrigerated centrifuge is needed). Three to four 0.5 to 1-ml aliquots of serum should be placed in cryovials labeled with Study ID number, date, and ”serum”. As soon as possible these should be stored at -20°C until moved within 2 hours to -80°C for storage until shipment to KUMC.

6.3.3.2.2 CBC + Fasting plasma Comprehensive Metabolic Panel + plasma lignan levels

Two lavender top 4 ml EDTA plasma tubes should be drawn and one should be sent to a commercial lab for a CBC, the other should be centrifuged at ~400 x g for 15 minutes and two 0.5 to 1-ml plasma aliquots should be placed into cryovials labeled with Study ID, date, "plasma". These may be temporarily stored at 4°C but should be moved to -20°C within 1 hour and to -80°C within 2 hours of collection. **A 4 ml mint plasma separator tube** is also needed for the metabolic panel and should be sent to a commercial lab with the lavender top tube for CBC.

6.3.3.2.3 Fasting serum for Antimullerian hormone, IGF-1 and IGFBP3

One 4 ml gold top clot tube should be drawn, allowed to clot for 30 min at room temperature, and then centrifuged at ~2200 x g for 15 minutes. Place 0.5 to 1-ml aliquots into two cryovials and freeze at -20°C until transferred to -80°C within 2 hours.

The total tubes for this visit are two 4-ml red top, one 4-ml gold top, two 4-ml lavender top and one 4-ml mint top.

6.3.4 Buccal cell collection

It is possible that single nucleotide polymorphisms (SNPs) may affect biologic response or modulate lignan concentration (e.g., CYP17). Subjects will not be stratified by specific SNPs at randomization as this would interfere with efficient study conduct, but results will be utilized in a multivariate analysis assessing change in Ki-67and lignan levels. A DNA sample for assessment of SNPs will be obtained by rinsing with Scope® mouthwash and placing the effluent in containers provided by KUMC. Samples are stable in the mouthwash for years at room temperature with no special handling. Specimens will be shipped to the KUMC Central Collection Lab for logging and shipment to Intergenetics, Inc. for assessment by PCR-based methodology of a panel of SNPs thought to affect breast cancer risk and/or metabolic processing [114,115].

6.3.5 Lignan intake questionnaire

A lignan dietary/supplement questionnaire which we have tested in a premenopausal high risk population is based on a validated instrument by Thompson *et al*. [16,42], and queries intakes and amounts of ~125 foods containing significant amounts of lignans. This questionnaire, completed with a web based tool or on paper at the same time plasma and urine are obtained for lignans, will help us interpret unexplained increases in these specimens in women not randomized to SDG.

6.3.6 Quality of life questionnaires

A baseline symptom assessment will be made via the BCPT symptom checklist for overall measures of quality of life, the BPQ for breast pain and discomfort. The two questionnaires will be completed by subjects, with an estimated competition time of 30 minutes:

- BPQ breast pain questionnaire, a modified McGill Pain questionnaire [116],
- Breast Cancer Prevention Trial (BCPT) Symptom Checklist [117,118]

6.3.7 Menstrual diary

Women will continue to record days of menstrual bleeding or spotting throughout the trial.

6.3.8 Baseline symptom assessment

Symptoms (e.g. diarrhea, hot flashes, headaches) existing prior to initiation of study agent will be recorded using NCI common terminology criteria for adverse events version CTCAE 4.0. This will assist the on site coordinator in determining whether symptoms arising during the course of the study qualify as adverse events (new or worsening symptoms).

6.4 Evaluations During Study Intervention

6.4.1 Telephone contact

Subjects will be contacted by the study coordinator at months 1, 3, and 9, to review dosing, concomitant meds and adverse events. Subjects who are difficult to reach or do not respond to messages and other means of contact may be considered non-compliant. These will be recorded in the source documents and subsequently entered onto CRFs.

6.4.2 6-Month Intermediate Visit

After 6 months of study agent, a clinic visit will be scheduled to conduct an in-person review of

- Symptoms
- Compliance
- Concomitant Meds
- Adverse Events

The subject should bring all remaining study agent to the visit so that the number of capsules can be counted and then returned to the subject. An additional supply of study agent will be dispensed to the subject.

6.5 Evaluation at Off-Study 12 Month Visit (see section 6.1 for Study Calendar)

If necessary for synchronizing the scheduling of RPFNA with the menstrual cycle, study agent may be continued for up to two additional months, until the RPFNA is performed. If this occurs, then studies associated with the repeat RPFNA and completion of study will be conducted at that time, not at a calendar 12 months. Systemic use of antibiotics must be stopped 3 weeks prior to the off-study RPFNA procedure and for 3 weeks prior to collecting blood or urine specimens for the off-study assessment of lignan levels. The procedures may be delayed by up to two months if necessary to facilitate this.

For non-menstruating women (see section 4.2.4), hormone levels (estradiol, progesterone, and FSH) must be assayed 2-4 weeks prior to RPFNA in order to predict when to perform RPFNA so as to be in the follicular phase. Lab results and institutional normal ranges must be reviewed by the Protocol Chair, who will provide an acceptable time window within which to conduct the RPFNA.

6.5.1 Off-study procedures and evaluations

Subjects should take study agent the day of the off-study visit approximately 3-4 hours prior to the time of anticipated blood draw. Specimen collection, processing, and storage; procedures; and assessments will be performed as at baseline (see sections 6.2 and 6.3) and include:

- Physical exam (with clinical breast exam)

- RPFNA – note: subject may (should) eat breakfast after blood draws but prior to RPFNA
- Concomitant medications
- Adverse events

Any remaining supply of study agent will be returned by the subject, counted by the study coordinator and recorded, and returned to the Investigational Pharmacy. The assessment of compliance will be performed on the data entered onto the CRF from the pill counts.

6.5.2 Specimens collected for central laboratory assays

6.5.2.1 Urine collection for central lab assays

A spot urine sample should be collected during the clinic visit. Urine lignans are stable at room temperature for up to a week, but site personnel should decant two 12-ml aliquots in labeled 15 ml tubes as soon as possible and store at -80°C until shipment to KUMC. Site personnel should record urine volume as well as the time of collection.

Preferably, a 10-12 hour overnight urine specimen will also be collected and brought to the clinic. Site personnel should decant two 12-ml aliquots in labeled (Study ID, date, "urine") 15 ml tubes as soon as possible and store at -80°C until shipment to KUMC. Site personnel should record urine volume as well as the beginning and ending times of collection.

6.5.2.2 Fasting blood collection for central lab assays

Subjects should be fasting for at least 10 hours (but should take morning dose of Brevail for off study blood draw). The time of last food consumption and the time of collection should be recorded.

Two red top 4 ml serum clot tubes should be drawn, allowed to clot for 30 min at room temperature, and then centrifuged at ~3000 x g for 5 minutes (at 4º C, a refrigerated centrifuge is needed). Three 0.5 to 1-ml aliquots of serum should be placed in cryovials labeled with Study ID number, date, and "serum". As soon as possible these should be stored at -20°C until moved within 2 hours to -80°C for storage until shipment to KUMC.

One 4 ml lavender top EDTA plasma tube should be centrifuged at ~400 x g for 15 minutes and two 0.5 ml to 1-ml plasma aliquots processed into cryovials labeled with Study ID, date, "plasma". These may be temporarily stored at 4°C but should be moved to -20°C within 1 hour and to -80°C within 2 hours of collection.

Three 4 ml gold top gel clot tubes will be collected for estradiol, SHBG, testosterone and progesterone. Blood is allowed to clot for 30 minutes at room temperature and is then centrifuged at ~2200 x g for 15 minutes. A minimum of 0.5 ml to 1 ml of serum is placed in each of at least six cryovials pre-labeled with subject screening identifier, date, and type of specimen (e.g., serum). For non-menstruating women an additional 0.5 to 1.0 ml aliquot in one cryovial is required for confirmation of follicular phase. They are to be frozen at -20°C until can be transported to a -80°C freezer later that day for storage until they can be shipped to KUMC

An additional lavender 4 ml EDTA tube and **one 4 ml mint plasma separator tube** are needed for CBC and fasting metabolic chemistry panel performed the day of off study visit. If all off study visit blood is performed this day.

The total tubes for this visit are two 4-ml red top, three 4-ml gold top, two 4-ml lavender top and one 4-ml mint top; except for non-menstruating women for whom an additional one or two 4-ml gold top tubes are required.

6.5.3 Lignan intake questionnaire

The lignan dietary/supplement questionnaire which queries intakes and amounts of ~125 foods containing significant amounts of lignans. This questionnaire, completed with a paper copy or with a web based tool at the same time plasma and urine are obtained for lignans, will help us interpret unexplained increases in these specimens in women not randomized to SDG.

6.5.4 Quality of life questionnaires

A symptom assessment will be made via the BCPT symptom checklist for overall measures of quality of life, the BPQ for breast pain and discomfort. The two questionnaires will be completed by subjects, with an estimated competition time of 30 minutes:

- BPQ breast pain questionnaire, a modified McGill Pain questionnaire [116],
- Breast Cancer Prevention Trial (BCPT) Symptom Checklist [117,118]

6.5.5 Menstrual Diaries

Menstrual Diaries will be collected to document days of menstrual bleeding or spotting throughout the trial.

6.6 Post-intervention Follow-up Period

Approximately two weeks after last consumption of study agent, subject will be contacted by telephone by the trial coordinator to assess adverse events and answer any remaining questions.

7.0 BIOLOGIC SPECIMEN STORAGE AND SHIPPING

All specimens are stored in polypropylene tubes or cryovials labeled with subject initials, study ID number, date of collection, and contents of tube (screening specimens should use the screening ID number instead of the study ID number).

Tubes containing RPFNA specimens collected as in 6.2.3 should be placed on a rocker for 12-24 hours to ensure thorough bathing of cells with fixative. Specimen should be shipped the day after collection but not if that involves shipment on a weekend (in that case, ship on Monday). Tubes containing RPFNA specimens should be overnight shipped with cool packs and using the KUMC Outside RPFNA Shipping Form. The shipping form should also be communicated to the KUMC Lab with notification of the shipment. A call or e-mail should be made to make certain the lab is aware of the shipment.

Buccal cell specimens may be shipped in convenient batches at any time, using the same procedure as for RPFNA specimens: overnight, not on a weekend, with cool packs.

All baseline and off-study blood (serum and plasma aliquots) and urine specimens for a subject should be sent together, overnight, on 5 lbs or more of dry ice. Notification of KUMC via e-mail, fax (913-588-3821) or phone (913-588-3917) must be made to make certain the lab is aware of the shipment.

No specimens should be shipped the day before a holiday or weekend. If it is uncertain whether the KUMC lab will be staffed, contact them before shipping.

8.0 SAFETY ASSESSMENTS

8.1 Definition of an Adverse Event

An adverse event (AE) is any untoward medical occurrence in a study participant. An AE does not necessarily have a causal relationship with the treatment or study participant. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with participation in a study, whether or not related to that participation. This includes any death that may occur while a participant is on study.

Medical conditions/disease present before starting study treatment are only considered adverse events if they worsen after starting study treatment (any procedures specified in the protocol). For purposes of this study, only those events that occur after a subject is randomized are considered adverse events and should be noted as such. Abnormal laboratory values or test results constitute adverse events only if they induce clinical signs of symptoms or require therapy, and are recorded on the AE CRF under the signs, symptoms or diagnosis associated with them.

8.2 Adverse Event Reporting

8.2.1 Reportable adverse events

For this study, only subjects that have signed the study consent and have been randomized to study agent will have adverse events recorded.

8.2.2 Adverse event data elements

- AE onset date
- AE Verbatim Term
- CTCAE Term (version 4.0)
- AE end date
- Severity grade
- Attribution to study agent (relatedness)
- Reported as a Serious Adverse Event (SAE)?
- Action taken with study agent
- Outcome of the event
- Comments

8.2.3 Severity of adverse events

Identify the adverse event using the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. The CTCAE provides descriptive terminology and a grading scale for each adverse event listed. A copy of the CTCAE can be found at http://ctep.cancer.gov.

AEs will be assessed according to the CTCAE grade associated with the AE term. AEs that do not have a corresponding CTCAE term will be assessed according to their impact on the participant's ability to perform daily activities as follows:

GRADE	SEVERITY	DESCRIPTION
1	Mild	Barely noticeable, does not influence functioning Causing no limitations of usual activities
2	Moderate	Makes participant uncomfortable, influences functioning Causing some limitations of usual activities
3	Severe	Severe discomfort, treatment needed Severe and undesirable, causing inability to carry out usual activities
4	Life threatening	Immediate risk of death Life threatening or disabling
5	Fatal	Causes death of the participant

8.3 Assessment of Relationship of AE to Treatment

The possibility that the adverse event is related to study agent will be classified as one of the following: not related, unlikely, possible, probable, definite.

8.4 Follow-up of AEs

All AEs, including lab abnormalities that in the opinion of the investigator are clinically significant, will be followed according to good medical practices and documented as such, until such AEs resolve or become stable in the opinion of the investigator.

8.5 Serious Adverse Events

ICH Guidelines E2A and Fed. Reg. 62, Oct. 7, 1997 define serious adverse events as those events, occurring at any dose, which meet any of the following criteria:

- Results in death
- Is life threatening (note: the term life-threatening refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe).
- Requires inpatient hospitalization or prolongation of existing hospitalization
- Results in persistent or significant disability/incapacity
- Is a congenital abnormality/birth defect
- Events that may not meet these criteria, but which the investigator finds very unusual and/or potential serious, will also be report in the same manner.

8.6 Reporting Serious Adverse Events

8.6.1 Central Reporting

KUMC will report all SAEs to the KUMC IRB and to the FDA using appropriate reporting procedures established by institutional and regulatory guidelines.

All sub-contracted sites must report knowledge and information regarding an SAE to KUMC and the Protocol Chair or her designee within 72 hours of this occurrence via fax, email and/or telephone. A written report must be faxed to KUMC for review and possible submission to KUMC IRBI and the FDA, and to the local site IRB.

Attention: Carol Fabian, M.D.
Protocol Chair
cfabian@kumc.edu
FAX: 913-588-3679
Phone: 913-588-7791

8.6.2 Required information

Include the following information when contacting the Protocol Chair:

- Date and time of the SAE
- date and time of the SAE report
- name of reporter
- call back information
- affiliation/institution conducting the study
- protocol number
- title of protocol
- description of SAE, including attribution to study agent and expectedness
- subject randomization number

8.6.3 Follow-up of SAE

Site staff should send follow-up reports as requested when additional information is available. Additional information should be entered on the SAE form in the appropriate format. Follow-up information should be sent to the Protocol Chair at KUMC as soon as possible.

All SAE's will be followed until there is resolution or stability based on the PI's judgment.

9.0 BIOMARKER AND LABORATORY ASSESSMENTS

All biomarker and laboratory assessments conducted at the central laboratory at KUMC are described in Appendix 1.

Assessments conducted at consulting laboratories (lignan assays, polymorphisms, etc.) will be performed according to Standard Operating Procedures developed by each responsible site.

10.0 DATA MANAGEMENT

10.1 Source Documents and CRFs

Source documents will consist of CBC and chemistry profile clinical laboratory reports, and forms created specifically for this study that will capture information such as physical exam, phone contact information, adverse events, concomitant medications, demographics, etc. To the extent which possible, source documents (aside from reports) will be formatted to be similar to the CRF format, in order to make the transcription from source to CRF more reliable. Source document files/chart must be kept available at sites for monitoring visits during the conduct of the study through completion of data analysis.

Information for data analyses will be entered by site trial coordinators from source document onto Case Report Forms (CRFs). Data items from the CRFs will then be entered into the web-based CRIS study database.

Subsequently, the information entered into the database will be systematically checked by Data Management staff. Other errors or omissions will be entered on Data Query Forms, which will be returned to the site trial coordinators for resolution. Data queries must be addressed and returned to KUMC within 14 days of submission to site.

Laboratory data from RPFNA and blood analyses performed at KUMC or consulting sites will be entered in Excel spread sheets which can be merged with the database held at KUMC. The spread sheets, with confirming printouts, will be considered as source documentation.

All data collected for this trial will ultimately reside in a central database. There are appropriate checks (such as comparison of electronic files to validated paper printouts) on data accuracy and completeness for all sources of data. This is accomplished via inspection of summary reports prepared by the biostatistician for this project, Dr. Henry Yeh.

When the database has been declared to be complete and accurate, the database will be locked. Any changes to the database after that time can only be made by joint written agreement between the Protocol Chair, the Trial Statistician and the Data Manager.

10.2 Data and Safety Monitoring Plan

The Data Safety and Monitoring Committee (DSMC) of the University of Kansas Cancer Center will provide annual review of this trial
The DSMC monitors data management and subject safety. Subject accrual is reviewed annually by the Protocol Review and Monitoring Committee of the University of Kansas Cancer Center. The DSMC also provides advice on the overall conduct of the study. Meetings will be held on an annual basis. Interval conference calls may be conducted as required. The functions of the DSMC are primarily those of an overview nature in regard to both data and safety, as detailed below.

All data collected for this trial will ultimately reside in a central trial specific database. Data accuracy and completeness will be monitored via inspection of summary reports prepared for the DSMC by the biostatistician for this project.

The subject cohort consists of essentially healthy women who are defined as being at high risk for the development of breast cancer, but do not have any clinical evidence of or suspicion for an

existing malignancy. As such, side effects that might be due to the study intervention are expected to be minimal but should be easily identified and monitored.

In regard to adverse events (AEs) or serious adverse events (SAEs), it is not deemed necessary for the DSMC to address these for individual subjects. Rather, all grade 4 toxicities are reported as an SAE to the institutional review board immediately.

In addition to the functions performed by the DSMC, an External Advisory Board (EAB) will serve in a general advisory role in regards to assessing the progress that is being made on the study. Subject accrual rates, and specifically problems with accrual that might impact on the ability of the trial to be completed, can be addressed by the EAB. Likewise, the EAB may make suggestions on other aspects that would improve the quality of the trial. The EAB consists of two individuals with experience in clinical prevention trial: Sandhya Pruthi, M.D., of Mayo Clinic, Rochester, MN; and Kala Visvanathan, MBBS, of Johns Hopkins University, Baltimore, MD.

10.3 FDA Monitoring

The FDA may monitor/audit various aspects of the study. These monitors will be given access to facilities, databases, supplies and records to review and verify data pertinent to the study.

10.4 Record Retention

Clinical records for all participants, including CRFs, all source documentation (containing evidence to study eligibility, history and physical findings, laboratory data, results of consultations, etc.), as well as IRB records and other regulatory documentation will be retained by the Protocol Lead Investigator (Dr. Carol Fabian) in a secure storage facility in compliance with HIPAA, OHRP, FDA regulations and guidances, and NCI/DCP requirements unless the standard at the site is more stringent. The records will be maintained, at a minimum, for two (2) years after completion of the study. The records should be accessible for inspection and copying by authorized persons of the Food and Drug Administration.

11.0 STATISTICAL METHODS

The study is a Phase II, double-blind, randomized, placebo-controlled clinical trial to assess the potential efficacy of SDG as a chemopreventive agent, as measured by changes in proliferation index (Ki-67) between baseline and 12 months. Secondarily, the study will evaluate other pharmacodynamic properties of the agent and assess which biomarkers, if any, are modulated. Side effects, adverse events, and quality of life will also be assessed.

Standard randomization strategies will be employed. Institutional site and whether the woman is on or not on oral contraceptives will be the only stratification factors, so that each site can be provided with a unique randomization schedule at initiation. All eligible subjects will be randomly assigned to SDG or placebo in a planned 2:1 ratio.

11.1 Sample Size and Power

The primary outcome of interest is efficacy of SDG relative to the placebo, thus the pre- to post-treatment change in a proliferation index (percent of cells expressing Ki-67) is the basis of the power analyses. From our pilot study, the average percentage of cells expressing Ki-67 at baseline was 4%, and the average change in percent of cells expressing Ki-67 was a 2.4% reduction, for subjects meeting similar eligibility requirements as used in thistrial.

A 2:1 randomization of 220 evaluable subjects will provide 83% power to detect a 2.5% reduction in Ki-67 for the treatment group compared to no reduction in the control group; this will be our primary endpoint. This is based on an assumption of a common standard deviation of 6% for change in Ki-67 in both groups and a Type I error rate of 5%. Within the target of 220 evaluable subjects, we expect to recruit 72 women taking oral contraceptives (OC) and 148 women not taking OCs. Because the efficacy of SDG is unknown for OC users, we considered different scenarios and evaluated the average effect in the SDG arm and the corresponding power. For 0-70% efficacy of SDG among OC users, the respective average Ki-67 reduction range from 1.7% to 2.3% will result in a statistical power ranging from 50% to 76%.

Ki-67 reduction in SDG arm (non-OC)	Efficacy of SDG in OC	Ki-67 reduction in SDG arm (OC)	Ki-67 reduction in SDG arm (OC & non-OC avg.)	Power
2.5%	0%	0%	1.7%	50%
2.5%	20%	0.5%	1.8%	55%
2.5%	50%	1.3%	2.1%	68%
2.5%	70%	1.8%	2.3%	76%
2.5%	100%	2.5%	2.5%	83%

11.2 Statistical Analysis

All analyses will be intent-to-treat. All subjects randomized will be evaluable for safety and all subjects that have both a pre and post specimen are evaluable for efficacy. Primary analysis of efficacy will be performed on all evaluable subjects and secondary analysis will be done on those subjects who received at least 6 months of study medication and were confirmed as being in the follicular phase for both RPFNA procedures. From our experience we have very little missing data; however, if the missing data rate is above 5%, we will use multiple imputation to impute these values to perform the primary and secondary analyses.

The primary outcome will be change in Ki-67 (percent of cells expressing Ki-67) from baseline to 12 months. The two-sample t-test will be used to compare the change in Ki-67 between the SDG

and placebo groups. Any difference in baseline values of Ki-67 between the two groups would be due to chance, however, if there is a significant difference, then analysis of covariance adjusting for baseline values will be performed. Due to potential skewness in these variables we will assess whether or not the Central Limit Theorem may be assumed. Given the sample size the existence of an extremely high degree of skewness would require the use of the Wilcoxon Signed Rank test.

For the secondary endpoints, similar methods as above will be used to compare the treatment and control groups at two time points for all markers, i.e., serum hormones and clinical laboratory tests. We will also correlate change in the primary biomarker Ki-67 with change in other markers (e.g., bioavailable estradiol, cytokines, RTqPCR results) using Pearson's correlation coefficient. Again, if there exists a high degree of skewness in these variables, Spearman's correlation will be utilized.

Multiple regression analysis will also be used to investigate whether changes in Ki-67 are due to variables other than treatment effect (e.g., age, cytomorphology, Gail risk; as well as the predictive effect of other biomarkers on modulation of Ki-67). Best subsets and stepwise approaches will be employed while controlling for treatment group.

For comparison of moderate or worse toxicities we will consider grade 3 toxicity or premature trial discontinuation as the critical events. The prevalence of events in the SDG vs. the placebo arm will be examined by Fisher's exact test.

Evaluation of agent-induced hormonal changes - We will examine whether SDG has any effects on serum levels of bioavailable estradiol, and testosterone, determined at baseline and after 12 months of study medication. We will determine whether addition of SDG alters the frequency or length of menstrual cycles. Effects on follicular reserve will be evaluated with anti-mullerian hormone in a sub-cohort of pre-menopausal women between ages 21 and 35 at study entry. Antimullerian hormone (AMH) is one of the earliest and most sensitive measures of follicular reserve declines for approximately 10 years before the cessation of menses. A distinct advantage for trial purposes is that it does not vary with day of menstrual cycle [119].

Adverse Events and Quality of Life – Change from baseline to 12 months will be assessed for mastalgia (expected in 30-50% of subjects at baseline), menstrual cycle and follicular reserve, and quality of life, as a function of SDG intake, plasma lignan levels and urinary lignan excretion. We will use the breast pain questionnaire (BPQ), which is a modified short form of the McGill Pain questionnaire (SF-MPQ) to assess breast discomfort and the Breast Cancer Prevention Trials (BCPT) Symptom Checklist to monitor quality of life

Correlative analyses will be performed with plasma and urine ENL and total lignans as the independent variable, with modifying variables of baseline BMI age and specific SNPs such as CYP17.

Since this is a phase IIB trial, the power and sample size for the primary endpoint was determined with a two-sided type I error rate of 5%. All subsequent secondary, tertiary and exploratory analyses will also be tested with a two-sided type I error rate of 5%. This may result in an elevated false positive rate for these secondary and tertiary analyses, however, since this is a Phase IIB study, we felt to control the type I error rate for the secondary, tertiary and exploratory analyses would be too conservative.

12.0 REGULATORY

Any changes made to the protocol will be initiated by the Protocol Chair, and submitted to KUMC IRB (HSC) for approval. After KUMC IRB approval, collaborating sites will submit revised protocol changes to local IRBs for review.

The determination of the necessity to cease enrollment until revisions are approved will be determined by the Protocol Chair.

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Appendix 1 – Biomarkers and Testing Procedures

1. Measurement of Serum Hormones

Serum will be stored at -80°C until assay. Pre and post study samples will be run together to avoid batch variation.

Estradiol and SHBG will be assayed directly in serum by commercial kits from Diagnostics Biochem Canada Inc. The estradiol kit # CAN-E-430 (direct EIA); serum only) has a limit of detection of 10 pg/ml; intra-assay precision (CV) of 4.6-9.3%; inter-assay precision (CV) of 6.2-10.1%. SHBG (Direct ELISA) Diagnostics Biochem Canada Inc. kit # CAN-SHBG-4010 has a limit of detection of 0.1 nmol/L; intra-assay CV of 3.0-8.6%; inter-assay CV of 7.2-11.6%.

2. Measurement of Anti-Mullerian Hormone

Anti-Mullerian hormone will be assayed using commercial kits available from Beckman-Coulter (Brea, CA). Serum will be directly assayed using sandwich ELISA kits (#A16507); limit of detection is 1 pM (1 ng/ml = 7.14 pM), intra-assay CV ≤12.3%, inter-assay CV≤14.2%.

3. Measurement of Serum Adipokines, Cytokines,

Serum is collected in clinic, divided into 1-2 ml portions in cryovials and immediately frozen at -80°C prior to transfer to Dr. Hursting's Lab at University of Texas at Austin. Luminex Multiplex Cytokine Assays, adipokine Luminex Multiplex Endocrine Assay kits will be used according to kit directions.

4. Measurement of Serum IGF-I and IGFBP-3

IGF-I and IGFBP-3 will be performed from serum which has been stored frozen in aliquots at -80°C until analysis. Pre and post study samples will be run together. IGF-I and IGFBP-3 will be assayed by ELISA with the standard kits from R&D Systems (Minneapolis, MN). Aliquots of pooled sera will be used to assess inter and intra batch variation. Molar ratio of IGF-I/IGFBP-3 will be calculated as [.130 X IGF-I (mg/ml)]/[.036 X IGFBP-3 ng/ml].

The IGF-I kit (#DG100) is a sandwich ELISA kit with intra-assay CV 3.5-4.3%; inter-assay CV 7.5-8.3%; limit of detection 0.026 ng/ml.

The IGFBP-3 kit (# DGB300) is a sandwich ELISA kit with intra-assay CV 2.3-5.0%; inter-assay CV 5.4-8.0%; limit of detection 0.05 ng/ml.

5. Breast Tissue Assays

Fixed tissue will be stored at room temperature in the original collection tube of CytoLyt® for a minimum of 24 hours prior to transfer to PreservCyt®.in the Breast Cancer Prevention Laboratory. Cells may remain in PreservCyt® for up to 1 year before processing to micro-dissection for RNA harvest.

Frozen tissue in the cryovials will be pooled and aliquoted in the Breast Cancer Prevention Laboratory for later assessment of tissue, adipokines and cytokines via luminex, and phosphorylated intermediates of the PI3 kinase and AKT/mTOR using reverse phase proteomics. Cryovials are thawed in a cold tap water bath and immediately placed on ice. A 1000 µl pipettor is used to mix sample in each cryovial and pool all samples for 1 patient in a sterile snap-cap polypropylene tube on ice. An aliquot of a 10X cocktail of PhosSTOP phosphatase inhibitor (Roche Applied Science, Indianapolis, IN) and complete protease inhibitor (Roche) is added to bring the combined inhibitors to 1X. 250 µl samples are aliquoted into 4 or more cryovials and refrozen for retention or shipping to the responsible assay laboratory.

5.1 Breast Tissue Cytomorphology

Two slides are made from each pooled sample by a liquid-based technique (ThinPrep®): two for cytomorphology under RNAse-free conditions one of which will undergo microdissection, processing to RNA and then cDNA and frozen at -80°C until later use for RT-qPCR. Remaining cells will be left in PreservCyt® for later use for RT-qPCR. Initial cytomorphologic assessment is performed by the cytotech and then the slide is photographed and sent to the cytopathologist, Dr. Carola Zalles for final assessment. Slides will be given both a categorical classifier (non-proliferative or normal, hyperplasia, hyperplasia borderline atypia, atypical hyperplasia, or suspicious for cancer) and a Masood Index score [106]. The Masood score is the result of 1-4 points given for 6 different morphologic characteristics: cellular arrangement (monolayer, overlapping nuclei, clustering, loss of cohesion), pleomorphism, myoepithelial cells (many, moderate, few, absent); anisonucleosis (absent, mild, moderate, conspicuous); nucleoli (absent, micronucleoli, micro and rare macronucleoli, predominately macronuclei); and chromatin clumping (absent, rare, occasional, frequent). Change in Masood score will be assessed by subtracting the off-study score from the baseline score

5.2 Processing of Fixed Breast Tissue for RT-qPCR

Good quality RNA is obtained from Cytolyt® fixed cells when they are processed and Pap-stained under RNAse-free conditions if >500 epithelial cells are used and RNA is harvested within 72 hours of Pap-staining, or after a year or more if they are stored in the refrigerator unstained in PreservCyt® fixative [Petroff, 2006]. Following RNA isolation, the RNA undergoes one round of linear amplification, is converted to cDNA and stored at -80°C until assayed by RT-qPCR [122]. Currently 83% of LCM epithelial samples yield good quality RNA/cDNA using the above methods and we can reliably measure gene expression by RT-qPCR for a minimum of 25 genes from approximately one-quarter to one-third of the formalin-fixed aspirate. Specimens from PreservCyt® vials are first processed and Pap-stained under RNAse-free conditions and then checked by light microscopy to assure that similar types of cells are present on the slides for LCM as for cytomorphology and Ki-67. At least 500-1000 cells from the epithelial clusters on the ThinPrep® Slides are harvested by LCM within 72 hours of Pap-staining using an AutoPix™ Automated Laser Capture Microdissection System (MDS Analytical Technologies; Sunnyvale, CA). RNA is isolated using column based chromatography and treated with DNase to eliminate any residual DNA (RecoverAll™ Total Nucleic Acid Isolation kit, Ambion/Applied Biosystems). RNA is then linearly amplified using MessageAmp™ II RNA Amplification kit (Ambion/Applied Biosystems). The amplified aRNA is reverse-transcribed to cDNA using SMARTScribe Reverse Transcriptase (Clontech Laboratories, Inc., Mountain View, CA) primed with random nonamers. Quality of RNA/cDNA is determined by the QC Metric Assay [Petroff, 2006]. Primer/probe sets are designed, using Primer Express™ v 2.0, as close as possible (ideally within 300 bp) to the 3' end of each transcript, because the amplified RNA is 3'-biased.

5.3 Ki-67 Immunocytochemistry

For Ki-67 assessments, an extra slide will be made from PreservCyt® material and total epithelial cells will be delineated with Mayer's hematoxylin counterstain and proliferating cells with a MIB-1 (DAKO M7240) antibody and citrate buffer antigen retrieval. A DAKO automatic stainer is used for all stains to insure staining consistency. Manual counting is performed by two laboratory technicians. Hyperplastic ducts (if present) with the highest concentration of MIB-1 positively staining cells are selected for analysis and a minimum of 500 total ductal cells are scored. The consensus score is utilized for analysis.

5.4 Processing of Frozen Breast tissue for RT-qPCR

RNA isolation can be performed on fresh frozen tissue which has not undergone LCM. This is most useful for high abundance genes expressed in stroma and fat +/- epithelium such as adiponectin and leptin. A 250 µl aliquot of pooled thawed RPFNA specimen is removed and mixed with 750 µl TRIzol® LS (Invitrogen Corporation, Carlsbad, CA) at room temperature. After at least 5 minute incubation at room temperature the TRIzol® LS-sample mixture can be stored at -80°C for later RNA extraction. RNA is extracted from the TRIzol® LS-sample mixture according to the manufacturer's protocol, including glycogen (Invitrogen) as a carrier prior to RNA precipitation. RNA amplification, reverse transcription, and PCR analysis are performed in the same way as for formalin-fixed RPFNA samples [122].

5.5 Frozen Tissue Adipokine and Cytokine, Analysis

250 µl aliquots of frozen RPFNA will be labeled with date, HRBC #, and Study # and stored at -80°C until shipment to Dr. Stephen Hursting at the University of Texas at Austin for analysis via kits supplied by Luminex [120,121].

5.6 Frozen Tissue Reverse Phase Proteomics:

250 µl aliquots of frozen RPFNA will be labeled with date HRBC # and Study # and stored at -80°C until shipment to Dr. Gordon Mills at MD Anderson Cancer Center for immunoblotting with specific antibodies and analysis via ImageQuant with Viagene Software [123-125].

6. Assessment of Single Nucleotide Polymorphisms

Samples of buccal cells collected in Scope® mouthwash will be used to assay for single nucleotide polymorphisms,(SNPs) thought to be associated with influencing risk for developing breast cancer by virtue of modulating a variety of cellular and metabolic processes. Specifically, variants of CYP17 will be assayed since it has been hypothesized to affect lignin levels. Samples are stable in the mouthwash for months at room temperature and no special handling. Specimens will be shipped to Dr. Eldon Jupe at Intergenetics, in Oklahoma City, OK, for assessment [114,115], at an alternate laboratory if necessary, or will be assayed at KUMC.

7. Lignans

7.1 Measurement of Plasma Lignans

Fasting plasma will be shipped to Cincinnati Children's Hospital where it will be assessed by HPLC analysis with Waters Quattro Micro UPLC system coupled to electrospray tandem mass spectrometry (ESI-MS/MS) by Dr. Kenneth Setchell [111].

7.2 Measurement of Urine Lignans

Spot and overnight (12 hour) collections of urine will be shipped to Fred Hutchinson Cancer Research Center for measurement by Dr. Johanna Lampe using isotope dilution gas chromatography-mass spectrometry in SIM mode [41,112,113], with normalization by creatinine level.

8. Questionnaires

8.1 The BCPT symptom checklist is a 42 item list of problems common to peri and postmenopausal women and those undergoing anti-hormonal treatment in prevention trials. Women are asked if they have had the symptom in the past four weeks. If they answer yes, they are asked to grade the severity (0-4). This tool can be used to compute a total score or individual or related items of interest (e.g., three items relate to joint stiffness and arthralgias) may be scored [117,118].

8.2 The Breast Pain Questionnaire (BPQ), a modified short form of the McGill Pain questionnaire (SF-MPQ), will be used to assess breast discomfort and a possible effect of SDG to ameliorate symptoms of mastalgia [116].

8.3 Changes in menstrual cycle length will be assessed by having subjects complete a menstrual diary for each month they are on study.

8.4 Lignan intake will be assessed at study entry using a questionnaire modified after that developed by Thompson et al. [16,42] which queries intakes and amounts of ~125 foods containing significant amounts of SECO, pinoresinol, matairesinol, and sesamin, all of which can be converted to the mammalian lignans ENL and EDL [16].