Research Protocol (revised July 7, 2014)

Project Title: Mammographic Density and Soy Isoflavones

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Abstract. Populations consuming high levels of soy, as in many Asian countries, have lower levels of ovarian hormones, lower rates of breast cancer, and reduced mammographic density than populations consuming typical Western diets. These observations may in part be explained by our clinical studies that found ovarian hormone levels of premenopausal women to be lowest while they were consuming a diet that provided 15% energy from soymilk containing weakly estrogenic isoflavones. No adverse effects were observed. Further questions are whether isoflavones are the critical components influencing breast cancer risk and whether they do so by affecting other reversible risk markers for breast cancer, such as breast density. Women with dense breasts have a 4-6 fold excess risk of developing breast cancer. Moreover, greater breast density makes it more difficult to detect early breast cancer by mammography. To test the hypothesis that consumption of soy isoflavones reduces breast density, we propose a randomized, double-blind study, with two arms, and 100 premenopausal women in each arm. Women will be recruited and randomly allocated to take soy isoflavone pills (treatment) or placebo pills containing no isoflavones (control) for 2 years. The daily dose will be 120 mg of total isoflavones. Both treatment and placebo pills will contain multi-vitamin and minerals. Women will provide blood and urine samples before and during the supplement intervention period for the analyses of ovarian hormones and isoflavones. At baseline and after the intervention period, subjects will be examined for breast density by mammography and magnetic resonance imaging. The efficacy of the intervention will be determined by comparing mean changes of dense breast tissue over the 2-year intervention period in the two groups with adjustment for baseline values and individual patient characteristics of interest. We hypothesize that 1 to 2 years of a soy isoflavone supplement in premenopausal women will reduce the effects of endogenous estrogens and reduce progesterone levels further, which will explain a corresponding decrease in breast density. Our research results will have significant implications for breast cancer risk reduction. Reducing breast density can be expected to reduce the volume of target tissue at risk for breast cancer development and will improve the sensitivity of mammography for screening. The study may lead to a novel, non-invasive and economical approach to breast cancer prevention.

Introduction: In two separate soy feeding studies, we have successfully lowered not only levels of estrogens (by 25-40%) but also progesterone (by 40%) in all 16 premenopausal women after only 1 month of soy feeding (1-5). In these prior studies, only 400 kilocalories of the daily energy intakes (~15%) of each subject was replaced by soymilk and the isoflavone intakes were 100-200 mg (as aglycones) per day. Soy feeding also lowered estrone sulfate by 15% and androstenedione by 20% in 12 postmenopausal women after 4 months. In premenopausal woman, soymilk with or without isoflavones

was highly effective in lowering estrogens and progesterone. However, a soy diet with higher level of isoflavones decreased progesterone levels further compared to a soy diet without isoflavones. The isoflavone-rich soy diet also increased urinary excretion of the "anticarcinogenic" 2-hydroxyestrone without affecting the excretion of the "carcinogenic" 16α-hydroxyestrone. Results from statistical models suggest that the amount and the source of protein and isoflavones are main factors that modify ovarian hormone levels. In this proposal, we will determine the roles of isoflavones on breast cancer risk reduction by analyzing their influences on breast and bone density. No adverse reproductive effects (e.g. amenorrhea in premenopausal women or thickened endometrium and increased vaginal cornification in postmenopausal women) have been observed in our studies. The magnitude of changes in the ovarian steroids in our study was similar to the differences of these steroids found in populations with widely different risks for breast cancer. Our results may explain why Asian women consuming soy have a lower risk for breast cancer than women consuming western diets. Our results, therefore, have physiologic significance and implications for breast cancer prevention.

Objective: Further important questions are whether isoflavones are the active component(s) and whether isoflavones can favorably influence other known and preventable risk factors, such as breast density. Breast tissue consists of radiologically dense fibroglandular tissue and radiologically translucent adipose tissue. Breast cancer originates in fibroglandular tissue. Women with higher breast density have a 4-6 fold higher risk of developing breast cancer; moreover, greater breast density makes it harder to detect early breast cancer by mammography ("masking effect"). However, unlike many other reproductive risk factors for breast cancer, such as ages of menarche, menopause and first full term pregnancy, increased breast density is a risk factor that can be reversed. Breast density is, therefore, a potential measure of efficacy for preventive interventions. Moreover, breast density may reflect cumulative past exposure to endogenous ovarian steroids and, thus, be a better intermediate surrogate marker for breast cancer risk than, for example, short term monitoring of serum levels of ovarian steroids. We propose to test the hypothesis that continuous consumption of isoflavones beyond 1 month will reduce ovarian steroid hormones and lead to a corresponding reduction in breast density. Isoflavones bind to estrogen receptors with affinity and specificity differing from 17ß-estradiol. Because bone is also an estrogen responsive tissue, the effect of isoflavones on bone metabolism in premenopausal women cannot be easily predicted and should, therefore, be monitored and studied as proposed here.

Hypothesis/Aims: Isoflavones lower progesterone levels but have no immediate effects on circulating 17ß-estradiol. [Note: there could be a delay effect on 17ß-estradiol due to feedback regulation.] Because isoflavones bind to estrogen receptors (ERs), they can displace estrogens from ERs, thereby blocking the effects of endogenously formed estrogens on breast tissue without immediately affecting circulating levels of estrogens. If this occurs, the markers for studying such effects are breast density or bone density. The hypotheses to be tested are that chronic soy isoflavone consumption will decrease progesterone further and soy isoflavones will compete with 17ß-estradiol for binding to ERs, thereby diminishing the effect of estradiol on breast (and bone) tissue, leading to a corresponding decrease in breast density (and possibly bone density). We will determine:

Aim #1: effects of continuing exposure to soy isoflavone beyond 1 month on the time-dependence of the levels of ovarian steroid and other hormones
Aim #2: effects of chronic soy isoflavone ingestion on breast and bone density

Aim #3: the interactions of isoflavones and other nutrients on breast density using statistical models
Aim #4: the safety of chronic soy isoflavone ingestion

Experimental Design and Methods: These aims will be achieved by a prospective, parallel 2-arm, repeated measures, randomized, double blind, placebo controlled study. After a successful completion of a 2-month baseline observation, qualified women will be randomly allocated to either placebo pills (control) or 120 mg isoflavone pills (aglycones, treatment) 5 days per week for 2 years with blinding of subjects, research staff and investigators to the supplement assignments. Both treatment and placebo pills will contain required daily intake of multi-vitamin and minerals. The main outcomes are: 1) breast density by mammography and MRI, 2) bone density by dual energy x-ray absorptiometry (DEXA) and 3) serum, nipple aspirate, and urinary levels of estrogens, progesterone, and other breast cancer risk markers, and changes from baseline to intervention. The explanatory variables are nutrient intakes, isoflavone intakes, urinary and plasma levels of isoflavones, demographic data (body weight, height), and reproductive history. Breast (and bone) density is chosen as a major outcome of interest because it is an index of cumulative exposure to ovarian hormones and is postulated to be intermediate biomarkers for breast cancer risk (6). To monitor safety, blood chemistries including liver and thyroid function tests, lipid profiles, blood cell counts, menstrual cycle lengths, and cytology of nipple fluid aspirates (to determine presence or absence of hyperplastic epithelial cells), endometrial histology, vaginal smear cytology, unexpected vaginal bleeding, breast lumps, and bone density will be monitored once every three months during all outpatient visits.

Inclusion/exclusion criteria: Women must be 30-40 years of age with regular cycles, FSH levels ≤ 10 mU/ml and mid-luteal phase progesterone > 8 ng/ml. Mammograms must be normal. Exclusions include use of contraceptive agents, other exogenous hormones, medications known to affect mammographic density, any medically prescribed diet, current pregnancy or lactation, previous history of cancer, breast cancer in a first degree relative, breast augmentation, reduction, or lifting, and peri- or postmenopausal status.

All procedures (mammography, MRI) and samples (fasting blood, overnight 12-hr urine, and nipple fluid) will be obtained during the mid-luteal phase of the menstrual cycle (between cycle days 20 to 24 preferably day 22) when progesterone levels peak. Subjects will record cycle day 1 (onset of menstrual spotting) throughout the study and complete a dietary habit history questionnaire (DAQ) (8) and reproductive history and general health history questionnaires (PHQF).

During each GCRC outpatient visit, subjects will bring a 12-hr overnight urine (stored in a urine jug containing sodium azide and glycerol), 24-hour food records. Menstrual cycle lengths and body weights will be recorded and fasting blood samples obtained for serum chemistries, liver and thyroid functions, lipid profiles, and steroid hormones. Breast fluid secretors will provide nipple fluid aspirates for analysis of epithelial cytology and hormones.

Baseline observations (2 menstrual cycles): There will be 3 GCRC visits per menstrual cycle for at least 2 cycles. Complete history, physical examination and a Pap smears will be obtained. Subjects who qualify will then be randomized, undergo mammography (a standard screening mammography and one extra view), bilateral breast MRI, and a bone density measurement by DEXA (L2 to L4 and hip).

Pill supplementation intervention period (24 months): Isoflavone or control pill supplements will begin on the second day of menstrual bleeding soon after the baseline observation period. Every three months (~ seasonally) subjects will visit the GCRC on day 22 of a cycle. At these visits, subjects will be provided with the assigned supplement pre-packaged for daily dose and consumption. Subjects will be randomly assigned to placebo or treatment in blocks of six to assure equal sizes of the pill group and of the sub-groups for blood sampling, using the PLAN procedure in SAS© (9).

If more than 6% of subjects (12 subjects) develop amenorrhea, we will break the code for these women. A transvaginal probe ultrasonography and an endometrial biopsy will be performed if amenorrhea occurs. If amenorrhea is related to soy isoflavone treatment, the study will be terminated. If this occurs, a statistically significant change in breast density is then expected because the progesterone levels of these women will be <1 ng/ml. The mammography, MRI and bone density measurements will be performed at yearly intervals and whenever subjects notify us of their intent to leave the study.

Statistical Analysis. The primary outcome variables are measures of breast density (mammographic density and relative volumes of parenchymal and adipose tissues by MRI). The main analysis will assess changes in breast density (BD) parameters over the 2-year follow-up period, measured at baseline, at one year and at two years. The efficacy analysis for the measures of BD will be analyzed primarily through mixed models for continuous outcome measures. This class of linear models can accommodate missing data and allows inclusion of time-dependent covariates for outcomes that are approximately normally distributed. Software is widely available (e.g., MIXED procedure in SAS®). We will model the marginal responses of the breast density parameters for each intervention group. The main effects of time, supplement group, and the (time x supplement group) interaction will be assessed. Time-dependent covariates (e.g., BMI) and time-independent variables (e.g., parity, age of menarche, race/ethnicity), including potential confounding variables, can be added to the models. The analysis will proceed with an intent-to-treat principle in which any subject randomized to a treatment group (i.e., placebo or soy isoflavones) with at least one post-baseline BD measure will be included in the statistical analyses. Some analyses will be conducted which will include only those patients with complete data up to a designated data collection time point (e.g., 12 months, 24 months, etc).

Power analysis and sample size justification. The main assessment of outcome is the change from baseline in percent dense tissue. The hypothesized mean change in area of dense tissue in the soy isoflavone group will be a decrease between 10 and 15 percentage points on average, while the placebo group will remain about the same (6; 10-15). Using a two group t-test with a 0.05 two-sided significance level, and estimates of the SDs that range from 20 to 10, we will have 80% power to detect changes in mean percent of dense tissue of –10% to –20%. If we experience a 20 to 30% dropout rate, the study can still detect changes of –10% if SD ≤ 20, and –15% or larger for SD ≤ 25. Our choice of 100 patients per arm is robust to any violations of our assumptions, for example unanticipated study variation or higher dropout rates than expected. Additionally, we can expect the study to have even greater power than we estimate since the proposed analyses (ANCOVA, mixed models) exhibit greater power characteristics than the t-tests upon which our power analysis is based. With an estimated attrition rate of 15% over the length of the study, 100 subjects per arm should provide 85 evaluable subjects at the completion of the study. We will recruit until 200 women complete at least 1.5 years of supplement.

Results expected. Increasing the duration of consumption of soy isoflavones is expected to decrease circulating (and nipple fluid) ovarian steroids, mammographic

density, and volume of breast with parenchymal tissue relative to adipose tissue. We expect the rates of these decreases to be greater in the isoflavone group than in the placebo group. Proliferation markers in nipple aspirates and the presence of hyperplastic epithelial cells in nipple aspirates may indicate the degree of stimulatory effects of isoflavones on breast tissues. This study will show whether breast MRI which measures 3-D features of the breasts may be more sensitive in detecting breast density changes than mammography, which is a 2-D assessment. Ability to use MRI (no radiation exposure) to measure breast density will allow us to assess in the future whether soy pill supplementation intervention on breast density and breast cancer prevention may be more effective if initiated during puberty than during adulthood, as data from animal studies have shown. The effect of isoflavones on bone is hard to predict and will be examined in this study.

Significance. Our results will have significant implications for breast cancer risk reduction, because reducing breast density can reduce breast cancer risk by two mechanisms: 1) by reducing the volume of target tissue at risk, and 2) by improving the sensitivity of screening mammography for early detection of breast cancer. The study will provide needed pilot data, to clarify in the future, whether an intervention that reduces breast density is likely to lead to a corresponding reduction in breast cancer incidence. A novel, non-invasive and economical approach to breast cancer prevention may be provided by the results of this study.

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Methods: We have extensive experience measuring serum levels of ovarian steroids, serum proteins (SHBG, growth factors, prolactin, cytokines, estrogen receptor etc), and isoflavones in human studies. These measurements will be by either radioimmuno- or enzyme-linked immunosorbent assays using commercial kits. An overnight urine will be collected for 12 hours in a one-liter urine jug which contains sodium azide and glycerol) by the study subject before each scheduled GCRC visit. The urine will be analyzed for isoflavones to assess other soy exposure and for steroid metabolites such as oxidative estrogen metabolites by either immunoassays or gas chromatographic methods.

Soy isoflavone pills and maltodextrin placebo pills. The make-up of the isoflavones is the same as that which exists naturally in soy. We believe studying the effect of the two soy isoflavones at their naturally occurring ratio is a pre-requisite to contemplating a study that addresses the effect of different ratio or the individual isoflavone. The total amount of isoflavones in each tablet will be maintained constant through formulation. The formulation consists of, for example, 125 mg Novasoy (50 mg of total isoflavones as aglycones) or equivalent weight of placebo material, 60 mg sorbitol, 3 mg magnesium stearate, 412 mg dicalcium phosphate with a total of 600 mg per tablet. [Note: Volunteers in this study will take 2 pills (=120 mg isoflavones as aglycones) per day.] For placebo, 10-DE maltodextrin (90% weight) and RT-175 caramel color (10% by weight) will be substituted for Novasoy [Note: Volunteers will take 2 tablets per day.] The relative amount of genistein/genistin to daidzein/daidzin does vary between lots of soybeans and this variation is a natural phenomenon. This ratio for Novasoy has varied between 1.1 to 1.3 (mean ± SD: 1.2 ± 0.1) from lot-to-lot. However, we will use only one lot of Novasoy for the entire study. Therefore, the relative amount of the two isoflavones will be constant. All subjects (both treatment and placebo) will be given required daily intake of multi-vitamin and minerals. Vitamin pill will be packaged with treatment assignment pill in container for daily dosage and consumption.

Breast density: Breast density of subjects will be determined by both mammography and bilateral breast MRI at the outpatient Radiology Clinic at UTMB. Both tests will be performed during the luteal phase, as confirmed by obtaining a blood sample on the days of the breast density tests for measuring progesterone.

MRI is superior to mammography for measuring parenchymal pattern (dense tissue) of the breasts, because density measurement by MRI is more objective, and thus more accurate, than the subjective scoring in mammography. Moreover, MRI does not require breast compression and does not involve radiation exposure, which will make it easier to study density changes in younger women in the future. Hence from the scientific point of view, MRI as an outcome measure is preferable. However, for practical translation of our study results to large populations, mammography as an end point for measuring density changes in population-based interventions is more feasible than MRI. The cost of mammography is approximately one-eighth that of MRI, and is customarily covered by insurance. Furthermore, most clinics are better equipped to perform mammography than breast MRI. Hence the efficacy of our pill supplementation interventions will be measured by two outcome measures, one of which will maximize scientific value, and the other to allow our results to be more readily compared to other reports and be more readily translated to standard clinical care.

Mammography: This test will be performed with a digital mammographic unit, a Senographe 2000D, S30311AR, a new Full Field Digital Mammography System (GE Medical Systems, Milwaukee, Wisconsin 53201). A routine quality control program includes sensitometric evaluation, phantom imaging, and regular visit by a medical physicist (Dr. Johnson). The instrument setting and breast compression will be duplicated and maintained for each woman for baseline and intervention mammography. The operator for the mammography will have the pre-diet imaging parameters for each woman on hand as a reference for all mammography. Screening mammograms include 4 views, 1 craniocaudal and 1 mediolateral views for each breast. Duplicate craniocaudal view will be performed for left breast, i.e. a total of 5 views for the study per subject.

One craniocaudal and one mediolateral digitized mammographic images will be randomly selected from each breast. Within each subject, the baseline and intervention period films will be matched for view as well as for breast. Quantification will be performed by subjective visual scoring using BI-RADS as well as by objective computer-assisted automatic measurement patterned after that described by Boyd et al. with modification.. Digitized mammographic images from each subject before and after pill intervention will be coded in random order, networked to a personal computer for image processing and interpretation by a radiologist for independent scoring using BI-RADS. Radiologist will have no knowledge of pill assignment or dates of mammography and will determine the density using BI-RADS^{TM} and rank the relative densities of images from baseline and intervention (categorical variable). The BI-RADS categories are: 1) entirely fatty (<25% dense area), 2) fatty with scattered fibroglandular tissue (25-50% dense area), 3) heterogeneously dense (50-75% dense area), and 4) extremely dense (>75% dense area).

The digitized images of these mammograms will be used for measuring areas of dense and non-dense tissues using a free image analysis software ImageTool. This software uses a histogram segmentation to assess dense tissue. Briefly, the reader selects a grayscale threshold value differentiating the entire breast tissue and a second threshold delineating only the dense tissue. Binary masks are made from each threshold value (the

mask is all pixels with the selected intensity and higher), the pixels in the mask counted, and the percentage dense tissue calculated as a percentage. Readers (Drs. Brunder and Lu have both been trained by Dr. Khamapirad on density determination.) will be blinded to the pill assignment condition of each mammogram. These processes will be carried out on a PC. This histogram segmentation algorithm provides quantification of the area representing dense tissue (pixels isolated by the threshold intensity) and the area of the total breast in the mammogram. The number of pixels with intensities in the dense range will be expressed as a percentage of the total pixels in the breast tissue to minimize errors due to tissue determination. Statistical information for the dense and non-dense tissue also is available: mean and standard deviation of the pixel intensity. This process can be extended by using custom image analysis software to allow for normalization of the images, fitting of normal distributions to the breast histograms and estimation of volume averaging on the results. Breast area, dense breast area, and %-area with dense breast are parameters reflecting mammographic features to be used to measure the efficacy of the study's pill interventions. Percentage change in these mammographic features from baseline will be evaluated twice during pill interventions. Agreement between scoring by BI-RADS and by ImageTool for density rating will be evaluated.

MRI: MRI will be performed during the luteal phase of the menstrual cycle to control for small but detectable cyclical variations in breast density. MRI examination will be performed using GE Signa 1.5 Tesla MRI system. The study subject will be placed prone in the magnet with chest support to permit comfortable and unencumbered suspension of the breasts for imaging. In order to assure optimal MR signal uniformity, the breast will be imaged using the body coil. Coronal and axial multiple slices will be recorded to visualize the entire volume of the breasts. The proton density with and without fat suppression MRI images will be acquired for image analysis. The MR images will be networked to a version 4.0 GE Advantage Windows Workstation for processing and analysis of the breast tissues. A threshold algorithm will be applied to each image of the breasts to segment (separate) dense tissue. Using a 3-D image reconstruction algorithm, the multiple slices through the breast will result in a volume measurement of the entire breast and the dense tissue. In addition, a histogram function will be used to display the MR tissue signal distribution (mean and standard deviation) of the tissue type. Drs. Khamapirad and Johnson jointly will determine the appropriate threshold values to segment the dense tissues and the edge of the breasts. The volume of the entire breast, the dense tissue volume, and %-volume with dense breast are main outcomes of interest for statistical analysis.

Nipple fluid aspiration: Because several studies showed that isoflavones may be stimulatory to breast tissues, nipple fluid aspirates will be studied to help understand the mechanisms by which soy isoflavones may reduce breast density and have any affect on breast cell proliferation. A technique to aspirate breast nipple fluid from non-lactating women has been established in our laboratory. After compressing their breasts gently from all four quadrants, the

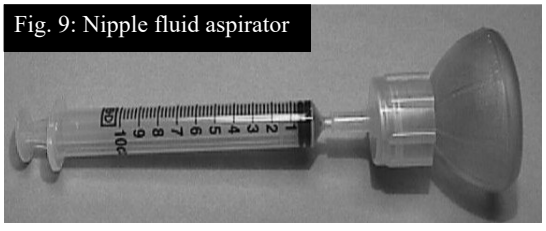
Fig. 9: Nipple fluid aspirator

subjects use a custom-made breast cup (basically a plastic funnel, Fig. 9) that is connected to a 10 ml-syringe. To apply suction, the plunger is pulled back to the 7-ml mark on the syringe and held at this pressure for 15 sec. If there is a keratin plug in the nipple, several initial suctions may be necessary to remove it. Once the keratin plug is removed, breast fluid can be recovered into a 200 μl-capillary tube by applying negative pressures described above. The volume is measured (1 mm in capillary tube = 1 μl). A portion of the nipple fluid is used for cytological evaluation for epithelial cells, and the remaining fluid transferred to phosphate buffer saline and stored at –80ºC for later biochemical analysis. We will obtain fluids from secretors 4 times per year and make similar number of attempt to obtain fluid from non-secretors (8 times total during two year intervention). Repeated breast fluid samplings may increase fluid secretion. To understand the effect of samplings on breast fluid volume, nipple aspiration will also be repeatedly performed from non-secretors.

BMD (bone mineral density) measurement: The BMD (gm/cm^2) in the lumbar spine (L_2 to L_4) and hip will be measured using QDR® 4500A Fan Beam X-Ray Bone Densitometer (Dual Energy X-ray Absorptiometry, QDR® 4500A Fan Beam X-Ray Bone Densitometer, Waltham, Mass.). The densitometer is calibrated daily using a phantom. Daily spine phantom quality control scans will be preformed and adjusted to within 1% of the reference value. The coefficients of variation for the anatomic phantoms will be maintained <0.5%. This method was sensitive enough to detect BMD changes in pre- and post-menopausal women during tamoxifen treatment and in PEPI trial to measure BMD changes in women after only 1 year of 0.625 mg conjugated equine estrogen treatment. QDR® 4500 is a high performance fan-beam densitometry. T score and Z score will be calculated using NHANES reference data and with adjustment for weight and ethnicity. QDR® 4500A dual femur application measures both femurs in one quick scan and calculates the mean BMD of both femurs. The average gives better precision for monitoring changes.

Soy isoflavones have been shown to stimulate osteoblast cell activities in vitro. In ovariectomized animals, soy isoflavones increase bone mineral density. Because the soy isoflavones, daidzein and genistein, are not pure anti-estrogens. Our study population is premenopausal women who generally have high levels of endogenous estrogens. It is not clear what would be the net bone effects of soy isoflavones in premenopausal women. Moreover, soy isoflavones may have bone sparing effects independent of their estrogenicity. Therefore, it is important to know if the extent of displacement of ovarian hormone by soy isoflavones may affect bone density. Second, women with high BMD were also found to be at higher risk for breast cancer. Information on BMD will allow us to assess if BMD may be a useful surrogate biomarker for estimating breast cancer risk in future breast cancer prevention studies.

All scans will be performed in duplicate with the participants in the supine position. The duplicate scan is performed after participant gets off and then returns to the densitometer. Identical anatomical regions of interest, total hip, femoral neck, Ward triangle, the trochantor, and the intertrochantor areas, will be scanned during repeat scans

using a software feature of the DEXA program. The mean of the two scans will be used for statistical comparison with results at other times in the study.

Study Population: Subjects must be 30-40 years of age and be free of mammographic abnormalities. They must be willing and able to return to Galveston for scheduled study visits. We expect to enroll the 200 subjects required for the study within 6-8 months. Women will be excluded from the study if they are on contraceptive agents, any other exogenous hormones, or medications known to affect mammographic density; are pregnant or lactating; or have a previous history of cancer, breast augmentation or reduction, abnormal mammograms, or are at high risk for breast cancer (first degree relative with breast cancer). Women who are on any medically prescribed diet, or are not willing to be randomized will be excluded. Women with pacemakers or other permanent metal implants such as IUD will be excluded from the study**.** During initial screening, past history of allergic reactions to foods will be acquired. If subjects have prior histories of food allergies to soy food, they will be excluded. Subjects must have regular cycles and mid-luteal phase progesterone > 8 ng/ml. Perimenopausal women with > 100 pg/ml of serum estradiol on cycle day 2 and > 20 mU/ml FSH will be excluded. Enrollment will be available to all racial groups. Pregnancy will be excluded by measuring urinary hCG (human chorionic gonadotropins) by standard clinical testing procedure.

Recruitment: The following methods will be used to recruit study subjects. 1) We have purchased 20,000 name records from a marketing company (KM Lists, Marlton, NJ). This initial list purchased contained only 35 to 40 years old women living within 30-mile radius of Galveston. We plan to mail study advertisement brochure to every woman in this mailing list. If we cannot recruit sufficient subjects, we will purchase name records for women age younger than 35 and older than 30. The database includes age, race, address, date of birth, income, education etc. 2) Advertisement materials have been distributed thru email systems of UTMB, area women's organizations, health networks, local charities, churches, and beauty saloons. In this procedure, we usually contact a person who maintains an email mailing list and ask this person to email our advertisement information to those in the list. 3) Study information and recruitment are also posted on websites or bulletin boards after securing permission from appropriate authorities. 4) The advertisement brochure is also included in the reminder letter sent to area women by the UTMB Radiology Department for scheduling screening mammography. [PI note: We have not used this method since most of the women in this list are outside the age range of our inclusion criteria.] 5) Advertisement brochure will be left in the waiting area of mammography clinics, in local churches, beauty saloons and health fairs. 6) We will also advertise study in radio, TV and newspaper, if needed. [PI note: We have not advertised in newspaper, radio or TV because we found these methods not to be cost-effective in the past. We may not need to use these modes of advertisement since we are recruiting well with mass mailing. We do not do face-to-face recruitment of subjects in mammography waiting area of mammogram clinics.]

KM Lists is a direct marketing company. KM Lists is the list broker (a major wholesaler of Axciom's database) and Axciom is the list owner. The Acxiom Infobase file is a compiled database made up from the white pages, voter registration, tax accessor

data, vehicle registration data, and many other sources. The Acxiom Corporation has a national database which includes approx. 116 million households. We purchased name records with age and gender restriction. According to KM Lists, the age information comes from household census, questionnaires, driver's licenses, credit bureau records, birth, high school and college records, warranty registrations, and insurance records. The gender information comes from Acxiom's proprietary conversion software. Both Acxiom and KM Lists rent the data out for many various direct mail/telemarketing campaigns and for research.

A similar telephone conversation is conducted for the potential subjects who telephone us and whom our staff telephones. The staff provides the purpose of the study, highlights the procedures involved, and describes the inclusion/exclusion criteria as detailed as the subjects wish to know. From this phone conversation, the subjects determine whether they want to come to UTMB for an inform consent visit. In general, most of the potential subjects call to find out if they are eligible and how they can be qualified for the study and to know more about the study procedures. No potential subjects would commit to come in for an inform consent visit unless they have sufficient information to assess that they will qualify for the study and know for sure that they like the study. If they are interested, they are then invited to come to the General Clinical Research Center of UTMB for a detailed description of the entire study as it stated in the consent form. During this visit, the content of the consent form is verbally presented to the study subject. After this presentation and answering their questions and concerns, the subject is encouraged to take time to read the consent form. If they are interested in the study, they sign the consent form. We then collect and record eligibility information. During this visit, the study schedule and remuneration chart included in the consent form is carefully described to subjects such that subjects understand our expectation of them and they understand what we will do for them. The subjects are given a signed copy of the consent form to take home and are encouraged to discuss the study with family member or friends. The subjects are informed in the consent form and during the consent visit that they can withdraw from the study anytime.

The subjects are given the Harvard DAQ, Personal Heath Questionnaire for Females (PHQF), and Menstrual Flow Chart and Supplement Intake Chart (MFC-DSC) to fill out. The PHQF is the standard of medical care forms to be filled out by patients visiting UTMB clinics including our GCRC, respectively. PHQF is the form used by UTMB Gynecology Clinic to collect reproductive history. The PHQF are reviewed by the physician investigators of this project as a part of history taking. Subjects can fill out these forms while at UTMB or they can take these forms home and fill out. For Spanish speaking only subjects, our Spanish speaking nurse from our GCRC or the Spanish speaking staff translate the questionnaires or forms and collect information by interview. The subjects also take urine jug, food records form home to fill out. The subjects are instructed on how to keep track of their cycle information. On the first day of their menstrual spotting (cycle day 1), they call our office to give us this date. From this date, we calculate when their luteal phase (post-ovulatory phase, the latter half of the menstrual cycle, usually cycle days 14 to 28) will be and days 20, 22 and 24 of their cycles. The

appointment is made for them to come in on these dates to have fasting blood draws and nipple fluid aspiration, to turn in their 12-hour urine collection and food records.

During this inform consent visit, the subjects are told that they may be removed from the study after certain diagnostic tests have been performed, for example, low progesterone level, abnormal mammograms, abnormal blood chemistries that cannot be resolved and if they are going through menopause.

The inform consent is performed by the authorized Study Coordinators or the PI. The PI visits and interacts with the study subjects frequently during their scheduled GCRC visits for clarifying study procedures, study purposes, and their concerns.

Post-intervention follow-up: The pills for both the placebo and isoflavone arms of the study contain multivitamins, including riboflavin and minerals. Riboflavin produces a bright yellow color in urine after intake. The study participants were blinded to treatment assignment per study protocol, but this would not prevent them from second guessing the study arm that they were assigned to. Our study participants probably would have noticed a change in their urine color whenever they ingested study pills, and this color change would indicate to the study team (we analyzed their urine) their degree of adherence to taking the study pills. Additionally, subjects might second guess the study arm that they were assigned to. The study subjects knew that they were taking multi-vitamins but were never told of a possible urine color change, and none of the subjects asked about this urine color change.

The purpose of this post-diet follow-up is to find out what these guesses were. We will email study participants who have received a dietary assignment. We would like each subject to choose one of these three answers in the email: (i) I believe that I took the placebo (sugar) pill, (ii) I believe that I took soy isoflavone pill, or (iii) I don't have an opinion about which of these I took. The frequency for each answer by treatment assignment and an interaction between treatment assignment and answer will be analyzed by ANOVA. We hypothesize that, due to the yellow coloring of their urine, a higher percentage of subjects would guess that they were in the active isoflavone treatment group, because most study participants prefer to be assigned to the active treatment group, if choice was allowed. This information will provide us with data about whether riboflavin can also be an effective masking agent for treatment group assignment in future clinical trials, not just as a tracer of compliance of taking study pills. Masking of treatment assignment would have an added benefit of balancing the participation behavior among study groups, in addition to randomization. We will send the questions initially by email and, if no response, by regular postal mail. We will request a dedicated email address from UTMB's Information Service for this specific purpose.

The survey questionnaire is not expected to change risk of participation.

Clinical Procedures:

The flow chart below outlines the study procedures and timeline in month (mo) for each subject:

Recruitment (mo 0) → Screening (mo 1) → Obtain baseline data (mo 2-3), give menstrual cycle calendar → Pregnancy test, followed by 1st mammography, DEXA bone density scan, and breast MRI → Randomization → Pill supplementation intervention (mo 4)→ Outpatient visits to the GCRC twice in an assigned month and repeat this outpatient visit once every three months (4 times per year) to obtain treatment data (mo 4 to 15)→ End of 1-year pill supplementation intervention (mo 15), review menstrual cycle calendar→ Pregnancy test followed by 2nd mammography, DEXA bone density test, and breast MRI (mo 15) → Pill supplementation intervention continues (mo 15)→ Outpatient visits to the GCRC twice in an assigned month and repeat this outpatient visit once every three months (4 times per year) to obtain treatment data (mo 15 to 26), review menstrual cycle calendar → End of 2-year pill supplementation intervention, end of study, pregnancy test, followed by 3rd mammography, DEXA bone density test, and breast MRI (mo 26)

1. Recruit volunteer subjects at UTMB Radiology Clinic or through TV, radio and newspaper advertisement and by mass mailing.
2. Invite potential subjects to UTMB GCRC for informed consent by the Study Coordinators or the PI. After obtaining written consent from the study subject, screening tests will be performed while as GCRC outpatient to determine eligibility.
3. Screening visits/Baseline visits: If the potential subjects reported that they have regular cycles and cycle lengths are in general longer than 24 days, they are unlikely to be peri-menopausal and their subsequent visits for these women are considered baseline visits. These visits will be scheduled during the luteal phase, i.e. 20 and 22 or 22 and 24 days after menstrual spotting depending on subject's cycle length. Cycle day 1 is the first day of menstrual spotting. The subjects are informed to call and report to us the date when they first detect menstrual spotting/bleeding as soon as possible and the staff will calculate when their cycle days 20, 22 and 24 will be and schedule their GCRC visits on these dates accordingly. The staff also checks with the subjects periodically to make sure that they do not forget to tell us the first day of their menstrual bleeding.

   For the older potential subjects (>39 years old) who have regular but short menstrual cycles e.g. ~20 days, they may be peri-menopausal women. For women suspected to be peri-menopausal, they are instructed to come to our GCRC for a blood draw (15 ml, 1 tablespoon) on the day after the first detection of menstrual spotting/bleeding. This blood will be analyzed for estradiol and FSH to determine if they are going through menopause and if they may qualify for the remaining study.

   After written consenting, the following information will be collected to determine their eligibility; some are collected soon after consent while others obtained after all baseline visits.

Subjects must be:

a) 30 to 40 years of age with normal mammograms, free of mammographic abnormalities,
b) have regular cycles, FSH levels ≤ 10 mU/ml and mid-luteal phase and progesterone > 8 ng/ml (to be confirmed during baseline observation period),
c) be able to return to Galveston for scheduled study visits, and
d) be premenopausal.

The subjects must not:

a) use contraceptive agents, any exogenous hormones, or medications known to affect the mammographic density,
b) be pregnant or lactating,
c) have a previous history of breast augmentation, reduction or lifting, abnormal mammograms, or at high risk for breast cancer (history of breast cancer, first degree relative with breast cancer),
d) on medically prescribed diets, or be willing to be randomized,
e) be peri-menopausal, e.g. estradiol >100 pg/ml on second day of their menstrual spotting and FSH >20 mU/ml (to be assessed by blood tests),
f) have a pacemakers or other permanent metal implants,
g) have a past history of allergic reaction to soy foods,
h) be postmenopausal, and
i) have bra cup size greater than 38DD and no less than A [PI note: We are using digital mammography and the detector plate of GE's Senographe 2000 is too small for bra cup size larger than 38DD].

The other screening tests include a complete history (including a self-administered PHQF), and a physical examination including gynecological examination, a breast examination, and a Pap smear, a three 24 hr-food records (record the 24 hr food intake before each scheduled GCRC visit, a total of three times), one blood sample (22 ml, 1.5 tablespoon) for blood chemistries, complete blood cell counts, liver and thyroid function tests, ferritin, and lipid levels and one blood sample (15 ml if suspected to be peri-menopausal) for ovarian hormones and FSH measurement. Nipple aspiration will be performed on all subjects during these baseline visits. After the consent visit, enrolled subjects are given Harvard DAQ, PHQF, food record form with instruction, a urine jug with sodium azide and glycerol added and instruction on how to collect 12 hour urine the night before each scheduled visit, and Menstrual Flow Chart and Supplement Intake Chart with instruction on how to record these information to take home.

During each GCRC visit (screening, baseline or pill intervention), weight, height, waist, hip, blood pressure, and temperature are recorded. These measurements are part of the standard of medical care. During each GCRC visit, any major changes in dietary habits, medications, and medical

conditions are checked and recorded. These changes may confound study outcomes and affect the continuing eligibility of subjects.

4. Screening and baseline visits. Subjects will record the first day of menstrual spotting (cycle day 1) and menstrual cycle lengths and visit GCRC on 2 cycle days of cycle days 20, 22, and 24 of two menstrual cycles. These dates of visits will be scheduled for the subjects by the staff based on the monthly report/phone calls by the study subject of the first day of menstrual bleeding. Subjects will bring back a 12-hr urine collected the night before each GCRC visit. Subjects will provide one fasting blood sample (30 ml for obtaining serum and 20 ml for isolating blood cells) during each of these visits. During every scheduled GCRC visit, the subjects will be queried if they have any major change in their dietary habit, medications, or medical conditions, and the changes, if any, will be recorded. Subjects will also bring back a 24 hr-food record describing what their food intake is for the 24 hr prior to the scheduled GCRC visit. Day 1 is the first day of menstrual bleeding. These blood samples will be analyzed for estradiol, progesterone, other steroids, and hormones. Blood cells will be analyzed for cDNA. Breast fluid secretors will also provide nipple aspirates during these GCRC visits. Attempts to aspirate fluids will also be made on non-secretors during these visits. Breast fluids will be stored and analyzed for selected steroids or hormones later. Subjects will bring back a 24-hr food record (24 hr food intake for the day before each scheduled GCRC visit) three times for each menstrual cycle. The 6 baseline blood samples are analyzed immediately after the subjects complete the baseline study for estradiol and progesterone. If they have regular cycles and if progesterone is >8 ng/ml, they will continue the pill intervention portion of the study. The subjects will be notified only if they are disqualified to continue. PI will call the to be-disqualified subjects and explain to them that their progesterone levels are lower than the cut-off to continue the study. We are expecting pill intervention to decrease progesterone levels. If their progesterone is too low to begin with, it may be very difficult to decrease it further by intervention. As to the clinical implication of a low progesterone level, the subject is advised by the PI to consult her personal physician.
5. Subjects who satisfactorily complete above tasks including the 4 scheduled GCRC visits will have a pregnancy test and, if not pregnant, will be given a mammography, a bilateral breast MRI, and duplicate DEXA bone density tests (L2 to L4 and hip) during one of these GCRC visits. The mammogram results will be interpreted immediately as for all clinical mammography tests by a licensed radiologist. Dr. T. Khamapirad (certified to interpret mammograms and breast MRI), our co-investigator will interpret the mammograms and if there are abnormalities, the subjects will be informed the test results by phone and by mail by the Radiology Staff according to the established clinical protocol in place in our Radiology Department. Specifically, the subjects will be advised to have additional diagnostic tests and be advised to consult their own physician for this follow-up. The cost of these follow-up diagnostic tests will not be the responsibility of this project. If the subjects have cancer lesions after further diagnostic tests, they will be excluded from the study. Dr. Khamapirad will inform Dr. Nagamani and the PI of any abnormal test results. PI will also phone

study subject, advise the subject to consult her personal physician, to have follow-up diagnostic tests, and to inform us the results of the diagnostic tests to determine their continuing eligibility in our study. Dr. Khamapirad will also assess the density of these women according to the established diagnostic guideline. Dr. Manubai Nagamani who is certified to interpret DEXA results will interpret bone density results for this project.

6. Pregnancy test will be determined during screening and during pill intervention if menstrual cycle lengthens by 2 weeks or more, or within 48 hours prior to scheduled mammography and DEXA bone density tests.
7. Subjects will be randomized to one of the two main supplement groups (isoflavone pills or placebo pills) and one of the three blood draw subgroups within each main pill group. There will be 1-day visits to GCRC for blood draw and other procedures on cycle day 22 of a specified cycle. Each of the subgroups within the main pill group will be randomly assigned to begin the 1-day period of blood drawing at 1-, 2- or 3-months, respectively, during initial randomization. For each GCRC visit (a total of 4 times per year) during the 2-year pill intervention, subjects will bring back the food intake records for the 24 hr preceding each scheduled visit, bring back an overnight 12-hr urine collection, fast overnight, provide fasting blood samples for the analyses of steroids and hormones, bring back MFC (with records of past day 1 of menstrual bleeding) and DSC (with records of supplement intake dates), bring back residual and unused supplement packets, provide nipple aspirates, respond to queries regarding major dietary habit, medical and medication changes, and have blood pressure, weight, height, waist, hip and temperature measurements. Subjects will take home another 3 months of assigned supplements, blank food record forms, urine jugs, MFC and DFC. During this 1-day visit once every 6 cycles, one blood sample will also be obtained for assessing blood cell counts, blood chemistries, liver and thyroid function, ferritin levels, and lipid profiles (monitor safety).
8. Subjects will be asked to mail back a 12-hr overnight urine 4 times per year. On a randomly assigned date, the staff will call the subject to collect a 12-hr urine and mail a specimen back.
9. The 1-day GCRC visit of an assigned menstrual cycle will be repeated once every three menstrual cycles (once every season).
10. Subjects will be provided with containers of the assigned pill supplement once every three months during one of these GCRC visits.
11. Subjects will consume the assigned pill supplements 5 days per week and be given a record book (Menstrual Flow Chart and Supplement Intake Chart) for recording study related information (day 1 of menstrual bleeding, GCRC visits, tests etc).
12. The Research Dietitian will obtain food records from study subjects on randomly assigned dates by phone. This will be done once every three months for each subject.
13. After completing 12 months of pill supplement, subjects will complete another Harvard DAQ (recall food intake frequency for the past 12 months of pill intervention) and will be given a pregnancy test, followed by a mammography, a breast MRI, and a DEXA bone density test.

14. Subjects will continue consumption of the assigned pill, have the 1-day visit to GCRC for blood draw and other study procedures once every three months (e.g. repeat steps 6 to 14) for another 12 months.
15. Pregnancy test will be performed whenever cycle length increases by >30% of each subject's usual length. Pregnancy test will also be performed no longer than 48 hrs prior to taking mammography and DEXA tests to ensure that pregnant women are not exposed to radiation. Gynecological examination including Pap smear will be done at the end of the study.
16. Transvaginal probe ultrasonography and endometrial biopsies will be performed when medically indicated, for example, if amenorrhea develops. This will be performed by Dr. M. Nagamani.

Risk and Benefit Assessment:

The soy isoflavone are currently available as pills over-the-counter and are found in soy foods sold to the public. We will use isoflavone tablets provided by the Archer Daniel Midland Co., which we have used in the past several years for several preliminary studies. These isoflavones are isolated by repeated alcohol extractions of soy protein isolates or soy protein concentrates (different processing). Alcohol is then removed by evaporation and the residues used for tablet formulation. In this way, the make-up of the isoflavones is the same as that which exists naturally in soy. These pills are sold as nutritional products over-the-counter to the public. The placebo pills contain maltodextrin, a carbohydrate, and should have no biological effect at the dose, 150 mg, to be given to the subjects.

Populations consuming soy have reduced risk of breast and endometrial cancers, reduced mammographic density, and reduced hip fracture rate. The FDA has approved a health claim regarding soy consumption and risk reduction for cardiovascular diseases. There are no other known risks from soy isoflavones from population-based studies, as soy products are commonly consumed in many countries. However, adverse effects have not been previously looked for carefully, so it is possible that there are unknown risks.

Isoflavones are weak estrogens and have beneficial such as acting as selective estrogen receptor modulators. However, in cell cultures or in animal models, the estrogenicity and biological effects of soy isoflavones are often concentration- or dose-dependent much like estrogens, are cell-type specific as well as species-dependent.

For example, anti-fertility effects have been observed in sheep but not in other species. Under low estrogen conditions, low dose of isoflavones stimulate the growth of hormone-dependent breast cancer cells in cultures, but high dose of isoflavones inhibit breast cancer cell proliferation. Isoflavones have also been shown to stimulate breast cancer cell growth in ovariectomized mice but the stimulatory effect was significantly less than that shown for the female estrogen, estradiol. In this xenograph mouse model, only one of the two isoflavones, genistein, is used and the free form (naturally occurring is conjugated form) is used which may not simulate human soy exposure experience. The breast cancer stimulatory effect of genistein shown in cell cultures and in xenograph mice

has not been found in humans yet.

Isoflavones cause thickening of the uterine lining in some animal studies (mostly in rats) after the ovaries were removed but this has not been found in sub-human primates. Moreover, our preliminary studies showed that 16 weeks of soy consumption at the dose that we plan to use for this study by postmenopausal women did not have estrogenic effect on the endometrium suggesting that it may not have adverse effect on the uterus. Other small human studies published to-date found no adverse estrogenic effect on the uterine lining from taking soy containing isoflavones or isoflavone pills. In fact, the only available epidemiological study indicated a lower rate of endometrial cancer with soy consumption.

Lowering of female hormones due to prolonged soy isoflavone ingestion may lower bone density, which could increase the risk of eventually developing osteoporosis (thin bones) and bone fractures. But this is not a known risk. Soy isoflavones have been shown to improve bone mineral density in animals. Asian populations consuming high levels of soy have lower rates of hip fracture compared to western countries. Because the current available information does not allow us to estimate the benefit and the risk of soy consumption on bone density, observations about bone is a part of this study. The risk of osteoporosis might also be increased if subject's calcium intake is not sufficient during the study. Subjects will be advised to adjust their calcium intake if needed to maintain an adequate intake.

Potential adverse effects such as lengthening of the menstrual cycle and nipple fluid cytology will be looked for closely during this study. If menstrual cycle lengths increase by more than 2 weeks in 12% of the participants (e.g. 12 subjects) for more than 3 cycles, the pill assignment codes for these women will be examined to assess if this is intervention-related. If this is associated with treatment, the ovarian hormones of these subjects will be immediately analyzed. The luteal progesterone levels in these women should be less than 1 ng/ml. If cycle length increase persists in these women for longer than 3 months, breast density in these women will be assessed first by breast MRI (because this involves no radiation exposure) and is likely to show changes. The pill supplement will be terminated in these women, but will be continued in the remaining study subjects. If cycle lengths increase in more than 30% of participants and this increase is study related, the pill intervention will be terminated and end of study mammography, bone density test by DEXA, and breast MRI will be performed.

If women develop amenorrhea, transvaginal probe ultrasonography and endometrial biopsies will be performed to ensure no adverse effects on the uterus. Manubai Nagamani, M.D. (a co-investigator) will prescribe and perform these tests. Study subject will be provided with a written pathological report for consultation with subject's own primary physician. Reports will also be provided to the Medical Monitor.

The total amount of blood drawn during each GCRC visit is 50 or 60 ml (3.3 or 4.0 tablespoons). Each subject will have a total of 12 GCRC visits for the collection of baseline and intervention data study. The total amount of blood drawn will be 46 tablespoons in 26 months. Blood chemistries, liver and thyroid function, lipid levels, and

serum ferritin levels of study subjects will be assessed once every 3 months during the study. If ferritin levels fall below 8 ng/ml, an iron supplement will be recommended. With blood drawing, there is a possibility of pain, bruising and infection. Blood draw will be performed by trained professionals.

Other than discomfort, there is no other known risk associated with nipple aspiration. If subjects cannot tolerate nipple aspiration procedure, this procedure may be omitted from these subjects. Nipple aspiration will be performed by trained professionals.

Mammography and DEXA are standard clinical tests. In these two tests, there is some radiation exposure. The amount of radiation exposure from mammography and bone density test will be equivalent to that of flying from Boston to Los Angeles. Radiation exposure may increase the risk of developing cancer. Mammography and bone density test will be performed by trained nuclear medical technologists.

Metal objects pose a serious risk for a MRI procedure because they can be affected by the high magnetic field. Surgical clips and other metal implants can be pulled out of place and devices such as pacemakers will not work in the magnetic field. Prior to MRI, all metal objects including jewelry are removed. MRI will be performed by trained nuclear medical technologists.

There is a possibility of cramping, bleeding, infection, and uterine perforation with endometrial biopsies. Most women have a few cramps in the pelvis after endometrial biopsy procedure that may last off and on for a couple of days. It is also common to have a small amount of bleeding from the vagina. The most serious potential risk from this test is "perforation" of the uterus. This occurs if the biopsy Pipelle is inserted too far, with too much force and it breaks through the wall of the uterus creating a hole. This is a problem because it can result in bleeding or infection, but this is not very common. One study found no infection and uterine perforation after 250 biopsies using Pipelle. Even without perforation, some patients can get an infection or have heavy bleeding that may require special treatment.

There is no known risk associated with vaginal probe ultrasonography. Vaginal probe ultrasonography, Pap smears, and endometrial biopsies are performed by a licensed gynecologist.

There is a possibility that samples provided during this study may be used in other research studies, or might have some commercial applicability that is not presently anticipated, and that this will not benefit the subjects. There is risk of loss of confidentiality. To avoid this, the information and samples provided to the investigators will be identified by a code number rather than by name. Preventive medical care including gynecological care will be provided to study subjects before and after the study and if needed.

There is a possibility of loss of confidentiality. To prevent loss of confidentiality, the information and samples provided to the investigators will be identified by a code number, rather than by the subject’s name. For additional precaution protecting against

loss of confidentiality, see the section "Disposition of Data". All key research personnel involved in this research have completed the required Human Subject Training available at www.utmb.edu/him/him_roi/confiden.htm.

There are no established benefits to the study.

Precaution, symptoms, and treatment of allergic reactions to soy: During initial screening, past history of allergic reactions to foods will be acquired. If subjects have prior histories of food allergies to soy containing food, they will be excluded. Symptoms associated with food allergies are diarrhea, fever and rashes. If subjects experience these allergy-like symptoms after pill intervention, they will be advised to stop the pill for several days until the symptoms disappear. If these symptoms recur upon resumption of pill, the subjects will be discontinued from the remaining study. Subjects develop allergic reactions during the study will be treated as clinically indicated by a physician investigators (Dr. Anderson or Nagamani) in this project. Subjects are also encouraged to consult their own primary physicians.

Cost of Participation.

The cost of tests done as part of the research, such as screening mammography, breast MRI, bone density test, screening Pap Smears, and screening tests for blood chemistries, liver function, lipid profile and thyroid function will be provided to the subjects at no cost. The costs of medical care and diagnostic tests (transvaginal probe ultrasound and endometrial biopsies) required for the evaluation of prolonged or absent menses and other adverse events possibly resulting from the pill supplement will also be provided to the subjects free of charge. The cost of follow-up diagnostic tests after an initial abnormal screening mammogram, such as spot compression and breast ultrasound, will be the subject's responsibility. The cost of HPV test after an abnormal Pap test result during the screening period will also be given free. These follow-up diagnostic tests after an abnormal screening test generally can be paid for by the subject's health insurance. The study pill will be provided free to the subjects. We will provide vitamins, iron and calcium supplements to all subjects regardless of pill assignment.

Remuneration.

Subject will be reimbursed for study visits to the GCRC for parking and travel expenses, and for time and inconvenience resulting from participating in the study. Subject will be informed of the amount of reimbursement before the study starts. Subject will be reimbursed after the completion of the first 4 baseline blood draw visits ($200 for all 4 visits or $50 per visit), and thereafter after the completion of every blood draw visit ($75 per visit per 3 months for the first year of pill supplement and $125 per visit every 3 months for the second year of pill supplement). If subject withdraw or become ineligible to continue prior to the completion of the study, subject will be reimbursed only up to the last completed blood draw visit. Other than medical care that may be provided and any other payment specifically stated in this consent form, there is no other compensation available for participation. Subject may be removed from the study, if subject cannot comply with the study procedures described in this consent form (for example, if subject do not take the pills, miss scheduled GCRC visits, blood draws and radiologic tests, or do

not provide food records, urine collections, menstrual records etc), if subject become pregnant, or if subject develop serious unexpected medical conditions.

Reporting of Serious and Unexpected Adverse Events:

Adverse experiences that are both serious and unexpected will be immediately reported by telephone within 24 hr and in writing within 3 days to: 1) UTMB IRB, 2) UTMB GCRC, and 3) UTMB Research Subject Advocate.

A UTMB IRB Adverse Event Report form will be used to report individual serious adverse event. For recurring adverse event, an aggregate report form will be generated to provide summary statistics for the study that will include resolution and potential protocol modification or study termination. The adverse event reports will include the name of P.I. (Lee-Jane W. Lu, Ph.D.) and co-investigators (Tuenchit Khamapirad, M.D., Karl E. Anderson, M.D., and Manubai Nagamani, M.D.), name of the study (Mammographic Density and Soy Isoflavones), UTMB IRB number (03-260) and UTMB GCRC protocol number (#635), the number of subjects enrolled to date, the number and type of serious and unexpected adverse events previously reported. The report of the medical monitor (Dr. Harold H. Sandstead) and a follow-up report describing the resolution of the adverse event will also be provided.

The expected adverse events are lengthening of menstrual cycle by more than 2 weeks per cycle in the absence of pregnancy for more than 3 cycles, amenorrhea, unexpected vaginal bleeding, breast lumps, abnormal endometrial histology/cytology, abnormal nipple aspirate cytology, and abnormal blood chemistries. Unanticipated adverse events are breast cancer, ovarian cancer, uterine cancer, intercurrent illness and intercurrent injuries. Serious adverse events are life threatening drug interaction, death, breach of confidentiality, and hospital admission.

Disposition of Data: All notebooks, Harvard DAQ, data collection forms, signed copies of the inform consent, and PHQF relating to this study will be kept in the locked filing cabinets on the 3rd floor of Ewing Hall, Division of Human Nutrition, Department of Preventive Medicine and Community Health, 700 Harborside Dr., Galveston, TX 77555-1109 where PI's office is located. The electronic data will be stored on the UTMB PMCH server (pmch on utmbf4) and the Bioinformatics Core facility of GCRC until published, and then archived in CD ROMs at the end of the study. The records will be kept for at least five years after the completion of publications resulting from this support.

During active data collection (e.g. screening, baseline and pill intervention periods), the data collection forms have subjects' names (to avoid making mistake), but these names will be wiped out from the forms and questionnaire and replaced with the assigned IDs as soon as the subjects complete the study. The PHQF is part of the subject's medical records and will be filed as such as soon as the subjects complete the study.

Each subject will be assigned a screening ID, BA001 to BA999 according to the

order they sign the inform consent which is also the order they enroll in the study. There may be dropouts during screening/baseline period but the ID of the dropouts once assigned will not be replaced. The subjects who complete the baseline study and if qualify to continue the pill intervention part of the study will be re-assigned another ID as PL001 to PL999 according to the order they begin the pill intervention portion of the study. Randomization will be made using PL IDs. The IDs are assigned by the Pharmacist and the key to the IDs is kept in the lock filing cabinets during active data collection. The Study Coordinators, PI, Dr. K.E. Anderson (co-investigator physician responsible for subjects' well-being), and the Medical Monitor have access to this key during this period. After the completion of study, the key will be kept at the locked filing cabinet at the PI's office and at this stage, the PI, Dr. Anderson and the Medical Monitor have access to the key.

Sample, image and record labeling: an example using subject PL001:

- Blood: PL001-S01 to PL001-S99 for sera, stored at –80ºC
- Blood cells: PL001-C01 to PL001-C99, stored in liquid nitrogen tank
- Urine: PL001-U01 to PL001-U99, collected in a jug containing sodium azide and glycerol, stored at –20ºC
- Nipple aspirate: PL001-N01(L,R) to PL001-N99(L,R); L, left breast; R, right breast, stored at –80ºC
- Mammography: PL001-MM1La,b,c,d, or e (left breast, a to e views), PL001-MM1Ra,b,c,d, or e (right breast, a to e views)
- MRI: PL001-MR1L (left breast), PL001-MR1R (right breast)
- Bone density: PL001-D01-L2, L3, or L4 (lumbar spine 2, 3 or 4), PL001-D01-H (hip)
- Food Records: PL001-FR01 to PL001-FR99

Locations and lengths of storage for collected specimen: These specimen upon collection will be temporary stored at –80ºC freezer at UTMB GCRC. Permanent storage will be at the –80ºC freezers on 3rd floor of Ewing Hall, 700 Harborside Dr., Galveston, TX 77555-1109. Specimen will be stored for at least five more years after publications of all study results.

All personnel listed below will have access to the particular portion of the data that they are personally involved in collecting and analyzing. For the purposes of patient care, certain co-investigators (Karl E. Anderson, M.D., Manubai Nagamani, M.D., and Tuenchit Khamapirad, M.D.) and the medical monitor will have access to patient-related medical information that is obtained during the study. Lee-Jane W. Lu, Ph.D. (PI) will have access to patient data that are important as study variables. Drs. Lu, Anderson, and the medical monitor will have access to the information linking subject's identity to their stored information. The Research Assistants will have access to research samples. All personnel involved in this project have been trained to maintain confidentiality of research and patient information. Information of medical nature including doctor's exams, PHQF, diagnostic test results, mammography and breast MRI results collected for conduct of this project will be entered into the subjects' UTMB medical records, as is customary and expected at this institution.

Modification of the Protocol: Protocol modification will be submitted to the UTMB IRB (03-260) and UTMB GCRC (Protocol #635) for approval prior to implementation.

Departure from the Protocol: Deviations from protocol that affect the well being of study subjects by investigators, staff or volunteers will be documented and reported to UTMB IRB and the GCRC. The P.I. will be responsible for reporting any such deviations. She will consult with members of the investigative team and discuss the impact and solution for such deviations. Minor deviations such as a missing blood draw or not taking one or a few pill supplements will be recorded in the study records but will not be reported.

Roles and Responsibility of Study Personnel:

Lee-Jane W. Lu, Ph.D. (P.I.): Responsible for all aspects of the study including study design, communicating with the UTMB IRB, UTMB GCRC, and NIH, recruiting subjects, obtaining informed consent on GCRC (if the Study Coordinator is not available) supervising the study and study personnel, consulting with co-investigators, and reporting and communicating study results.

Tuenchit Kamapirad, M.D. (Co-Investigators): Responsible for designing and interpreting radiological aspects of the study which includes identification of eligible subjects, supervising mammography, breast MRI, and bone density tests, and advising and training personnel on breast density determinations.

Manubai Nagamani, M.D. (Co-Investigator): Responsible for designing, supervising, and interpreting reproductive endocrinological aspects of the study which includes gynecological examination, assessing reproductive endocrinological eligibility of volunteers, and designing and supervising the analyses of steroids and hormones. Dr. Nagamani is certified to interpret bone density results.

Karl E. Anderson, M.D. (Co-Investigator): Responsible for designing and interpreting medical and nutritional aspects of the study which include determining eligibility of study subjects, providing medical care to study subjects, interpreting endocrinological test results, and supervising activities of the physician assistant.

Raleigh F. Johnson, Jr., Ph.D. and Thomas K. Nishino, Ph.D., (Co-Investigators): Responsible for designing, interpreting, and quality control for mammography, breast MRI and bone density tests which include parameter setting for radiological instrument and performing image analyses of mammograms and breast MRI images acquired for the study.

Donald G. Brunder, Ph.D. (Co-Investigator): Responsible for bioinformatic aspects of the study, including development and modification of software for breast density image analyses, network interfaces between the radiological instruments, PCs of investigators and staff, analysis of mammographic images, and designing electronic system for storing data acquired for the study.

Sadagopa Ramanujum, Ph.D. (Co Investigator). An analytical chemist, he specializes in

mass spectrometry, has developed methods for the analysis of isoflavones. He will be responsible for the analytical chemistry aspects of the work. This will include design methods and supervise the analyses of isoflavones and riboflavin.

James J. Grady, Dr. P.H. (Co-Investigator): Responsible for statistical aspects of the study including randomization of study subjects, designing methods for statistical analyses, supervising data management, interpreting statistical test results, and supervising statistical clerk and statistical programmer.

Rel Misti and Cynthia Stewart and to be named (Study Coordinators): Responsible for coordinating administrative aspects of the study, which includes advertising, recruiting subjects, obtaining informed consent on GCRC, scheduling outpatient visits to the GCRC and radiological tests, tracking subject compliance (supplement and protocol compliance), obtaining nipple aspirates, collecting, coding, and delivering data and samples to P.I. and co-investigators' facility for further processing.

Lara Powell-Gomez (Spanish Translation): Responsible for Spanish translation for Spanish-speaking only subjects.

Research Dietitian: Responsible for all dietary aspects of the work including providing dietary counseling to study subjects, recording dietary compliance, and nutrient analyses of food records and health habit history questionnaires. The Study Coordinators under the supervision of Ms. Ann Livengood (the Research Dietitian of UTMB GCRC) are responsible for collecting food records and food frequency questionnaire. Ms. Livengood is providing the counseling service.

The research pills will be dispensed by M'Linda H. Lasswell, RPh. M'Linda H. Laswell is the Coordinator, Investigational Drug Service, Department of Pharmacy, the University of Texas Medical Branch. M'Linda has performed randomization of investigational drugs for UTMB's clinical trials. She will dispense the pills for this study.

Research Nurse (to be named): Responsible for assisting Dr. Nagamani in performing histories and physical examinations of study subjects. .

Research Assistant (Neelam Bhopale): Responsible for aliquoting and cataloging blood, urine, and nipple aspirate samples, participating in obtaining nipple aspirates, analysis of isoflavones in urine samples, coding mammograms and breast MRI for blinded analyses, delivering data to statistical clerk for entry, and partial responsibility for assisting the Study Coordinator and Research Dietitian.

Yafei Huang (Graduate Student): Responsible for studying the effects of pills on protein profiles.

Xin Ma and Hua Cao (Research Assistant): Responsible for the analyses of steroids and hormones in blood and nipple aspirates and assisting in aliquoting and cataloging samples when needed.

To be named (Statistical Assistant, Office of Biostatistics): Responsible for developing

data collection forms, setting up an Access Database, inputting data, double data entry, quality control checks, file management, and generating monthly reports of study progress.

Statistical Programmer (to be named, MS): Responsible for creating SAS database for statistical analysis, creating tabulation, charts, and graphs of the data and program statistical analysis.

Medical Monitor (Harold H. Sandstead, M.D.): Responsible for monitoring the well-being of research subjects for study related conditions including adverse events and reviewing and reporting adverse events to UTMB IRB.