A PILOT STUDY OF HYDROXYTYROSOL, A COMPONENT OF OLIVE OIL FOR BREAST CANCER PREVENTION IN WOMEN AT HIGH RISK OF BREAST CANCER

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Table of contents

List of figures

List of tables

1 Background and Rationale

1.1 Overview of pathogenesis, epidemiology and current treatment

Breast cancer is the most frequently diagnosed cancer in women in developed countries. An estimated 226,000 new cases of invasive breast cancer and 39,920 deaths are expected to occur in 2012 in the United States [1]. Although the average lifetime risk of breast cancer in an American woman is one in eight, the risk is increased in women with a strong family history of breast cancer, BRCA 1 and 2 gene mutations, atypical hyperplasia or lobular carcinoma in situ (LCIS), and history of mantle irradiation for Hodgkin's disease[2]. Although new systemic treatments for established breast cancer has shown to reduce mortality[3],but the success rate is low. Major breakthroughs have come slowly, and greater attention is now being directed towards breast cancer prevention[4]. One particularly promising strategy has been to use medication to prevent breast cancer development.

Selective estrogen receptor modulators (SERMs)/Aromatase Inhibitors: The SERM, tamoxifen has been used in the treatment of breast cancer for many years. The observation that contralateral breast cancer was reduced in women treated with tamoxifen led to a number of chemoprevention trials with tamoxifen and eventually raloxifen. Results from such breast cancer prevention trials summarized in an overview analysis showed that SERMs significantly reduced breast cancer incidence by 38%[5]. Exemestane, an aromatase inhibitor evaluated in a double-blind, placebo controlled phase III study in 4560 postmenopausal women, demonstrated a 65% relative reduction in the annual incidence of invasive breast cancer as well as 53% decrease in the annual incidence of invasive plus noninvasive breast cancer[6]. Studies using selective estrogen receptor modulators, or SERMs, have shown that breast cancer prevention is feasible[5]. However, while breast cancer incidence is reduced by approximately 50% in these studies, not all cancers are prevented. First, there is no reduction of estrogen receptor (ER)-negative tumors. Secondly, even ER-positive tumors are only reduced by one-half to two-thirds. Because SERMs do not totally prevent breast cancer and because of the side effects of long-term SERM use, the majority of risk-eligible women are reluctant to take chronic antiestrogen therapy for primary prevention. Chronic therapy is necessary because these drugs suppress proliferation, but do not remove abnormal, premalignant cells. Thus, there is an urgent need to develop more effective strategies to prevent breast cancer that are tolerable to women at risk. Several nutritional studies have shown inverse relationship between the consumption of olive oil and the incidence of breast cancer[7]. Many of the beneficial properties of olive oil are associated with minor components of which hydroxytyrosol (HT) is the most promising.

The primary reasons for targeting pre and post-menopausal high-risk women are 1) epidemiological studies have suggested an association between increase in olive oil intake and reduction in breast cancer risk 2) Studies have shown a correlation between decrease in mammographic density and decrease in breast cancer risk in both pre and post menopausal women 3) there is a need for a compound that prevents all types of breast cancer and has less toxicity

Imaging and tissue-based biomarkers have been incorporated into this trial and as new information becomes available during the course of this trial, this study will provide a useful biorepository to draw from for biomarker discovery in future studies.

The purpose of this trial is to evaluate the effect of hydroxytyrosol on mammographic density in women at high risk of breast cancer, and secondarily to gain information concerning the ability of hyroxytyrosol to modulate the expression of breast tissue markers and serum markers.

1.2 Introduction to the investigational Product(s)

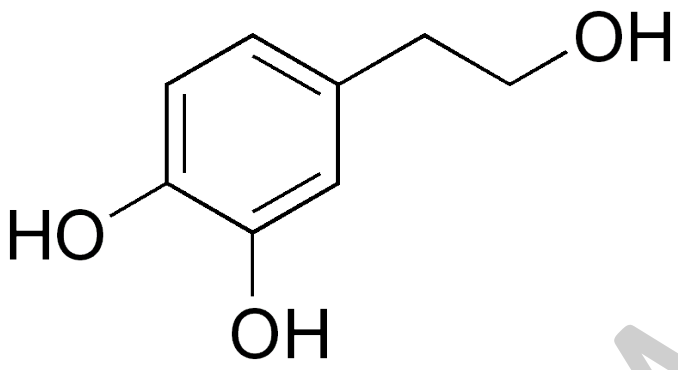
Hydroxytyrosol is a product of hydrolysis of oleuropein and due to its polar character it is found in great quantities in the remains from oil processing such as pomace olive oil, olive-mill waste water, or the rinse waters[8]. Preclinical studies have demonstrated HT to have anti-atherogenic, cardioprotective, anti-inflammatory, platelet aggregation inhibitor, and antimicrobial properties. This natural compound has been reported to regulate transcription factors (Nrf2, NFκB), cytokines (IL-6), prostaglandins (PGE2), COX-2, myeloperoxidase and response to stress through Wnt pathway[8-13]. Preclinical *in vivo* studies demonstrated the potent antioxidant HT to have an anti-neoplastic role by interacting with Wnt/sFRP signaling pathway.

Wnt/ β-catenin in Breast Cancer: Wnt/β-catenin signaling pathway is an important regulator of the stem cell pathway controlling the development of the mammary gland during embryogenesis. This pathway is also frequently found in invasive breast cancer and leads to a more aggressive phenotype and poor clinical outcome through the regulation of important cell events like proliferation, migration, differentiation, and apoptosis[14]. The sFRP family consists of five secreted glycoproteins (sFRP1-5) that function as Wnt pathway inhibitor by directly interacting with both Wnt ligands and FRP receptors. sFRP family are silenced in several types of cancers including breast cancer[14, 15]. The Wnt/ β-catenin pathway has also been implicated in the control of stem cells[16]. Recently, it has been demonstrated that sFRP4 generate ROS (Reactive Oxygen Species)[17] by blocking Wnt/β-catenin and resulting in oxidative stress. ***Effects of Hydroxytyrosol on Wnt pathway*:** The *in vivo* antitumor activity of HT was evaluated in an animal model of breast carcinogenesis[18], where the authors treated breast tumor-bearing rats with 0.5mg/kg HT for 5 days/week for 6weeks by oral gavage. They reported HT decreased tumor volumes in rat models to the same degree as treatment with adriamycin. HT also caused a marked decrease in tumor cell proliferation, and differential expression of genes related to apoptosis, cell proliferation, survival, and response to stress through inhibition of Wnt. Also a dose dependent decrease of β-catenin and cyclin D1 protein levels were observed. Similar findings were seen in epithelial to mesenchymal markers, a decrease in both Snail and Slug transcription factors and an increase in protein levels of epithelial marker E-Cadherin.

1.2.1 Preclinical Evidence for the Chemopreventive effects of HT

Antitumor *in vitro* studies have reported antiproliferative action due to overexpression of p21 and p27, and inhibition of CDK6, and pro-apoptotic events through activation of caspase-3. Moreover, regulation of NFκB, COX-2, and PGE2 are also involved in regulation of carcinogenesis attributed to HT[8-13, 18]. An *in vitro* study assessing HT effects on human promyelocytic leukemia cells and colon adenocarcinoma demonstrated that HT arrested cell cycle and induced apoptosis in tumor cells by arresting the cells in G0/G1 phase[19]. An *in vitro* study in MCF-7 human breast cancer cells demonstrated a similar finding of cell cycle arrest in G0/G1 phase resulting in a dose dependent growth inhibition[10].

Figure 1-1 Chemical structure of Hydroxytyrosol



1.2.1.1 Epidemiological Evidence for the Chemopreventive effects of Olive oil

In comparison with Northern European or other Western countries, Mediterranean countries have lower rates of mortality from cardiovascular disease and cancer. This is partially attributed to the so-called Mediterranean diet. Historically, scientific efforts have been focused on healthful benefits of olive oil as key element of a Mediterranean diet. Numerous preclinical, animal studies and at least six case-control and one cohort study has been conducted to test the health benefits of olive oil. A meta-analysis evaluating relative risk (RR) estimates for high as opposed to low olive oil consumption that demonstrated reduction of the incidence of breast cancer by 38% (RR 0.62 95% CI 0.44-0.88). One of the limitations for the meta-analysis was different definitions of the high olive oil consumption which ranged from two teaspoons a day, more than once a day, consuming 40.7g/day, or more than 8.8g/day. Despite that, it supports an inverse association between olive oil consumption and breast cancer risk[7]. Traditionally, many of the healthful properties associated with this edible oil have been ascribed to its high content MUFAs (monounsaturated fatty acids), such as oleic acid. Today, it is clear that many of the beneficial properties derived from the consumption of virgin olive oil are due to some of its minor compounds. The bulk of the research has focused on one of those compounds phenol hydroxytyrosol (HT).

1.2.1.2 Toxicology studies

Oral administration of a single gavage dose of solid olive-pulp extract at levels of 0, 1,000, 1,500 or 2,000 mg caused no adverse effects, except for soft or liquid feces. The rats were administered 5000mg/kg of olive pulp extract for 29 consecutive days and no mortality or clinical signs of toxicity were noted. The authors reported that the LD_{50} of solid olive-pulp extract was greater than 5g/kg (equivalent to 2.5-3.5g/kg of HT) suggesting that the extract is non toxic[20].

1.2.1.3 Clinical Studies in HT

In a multicenter, randomized, crossover, controlled trial of 200 healthy male volunteers were randomized to olive oils with high (hydroxytyrosol content 63.5mg/L, tyrosol 24.4mg/L, and oleuropein derivaties 327.2mg/L), moderate(hydroxytyrosol content 28.5mg/L) and low (no hydroxytyrosol) [21]. There was a linear increase in high-density lipoprotein (HDL) cholesterol levels with increasing in phenolic (hydroxytyrosol) content with the mean change of 0.025, 0.032 and 0.045mmol/L respectively. Triglyceride levels decreased by an average of 0.05mmol/L for all three levels of olive oil. Oxidative stress markers also decreased with increasing phenolic content [21, 22]. Oxidative damae to lipids was assessed by measuring plasma-circulating oxidized LDL, plasma total $F_{2\alpha}$-isoprostanes, plasma C18 hydroxy fatty acids (GC-MS) and serum LDL cholesterol un-induced conjugated dienes. There was a

significant linear decrease in biomarkers of lipid peroxidation (conjugated dienes, hydroxyl fatty acids and oxidated LDL) with increasing phenolic content of olive oils. Based on these data, the European Food Safety Authority (EFSA) felt that a cause and effect relationship has been established and approved 5mg hydroxytyrosol and its derivatives in olive oil should be consumed daily [23].

Two studies have evaluated the absorption of HT in healthy volunteers. The first study administered pure HT as a supplement in an aqueous solution (single oral dose of 2.5 mg/kg) in the plasma and urine of 10 healthy volunteers[24]. They found that the absorption of HT is rapid, with maximal plama concentration detected in 13 to 16 minutes and the levels were undetectable in 2 hours after administration. Another study administered 50ml olive oil in six healthy volunteers and demonstrated absorption of HT. The absorption was increased with sustained doses of olive oil [25]. An update in 2013 concluded a no observed adverse effects level (NOAEL) of 500mg/kg/day [26].

A dose of 25mg/day of HT was chosen due to following reasons 1) EFSA approved hydroxytyrosol as a healthy dietary compound up to 15mg/day. There is a previous unpublished study of using hydroxytyrosol 10mg BID in healthy volunteers with class I obesity (communication with PI). With this dose they did not see any changes in LDL oxidation levels and did not have any adverse effect. We used 25mg of hydroxytyrosol as there is demonstrated safety in previous studies and higher than usual healthy dietary compound.

2 Mammographic Density as a Marker of Breast Cancer Risk

Studies have consistently demonstrated a 4-6 fold increase in breast cancer risk for women with increased mammographic density for up to 8 years following measurement [25, 27, 28]. Breast density as assessed qualitatively by BI-RADS category (1= ―almost entirely fat, 2 = ―scattered fibroglandular densities, 3 = ―heterogeneously dense, 4 = ―extremely dense). Randomized studies have demonstrated that tamoxifen lowers mammographic breast density up to 4.3% yearly [29]. Given that tamoxifen has been shown to decrease the risk of breast cancer and does decrease breast density, density change may be a useful surrogate endpoint biomarker for the effect of tamoxifen and other chemopreventive agents on breast cancer incidence. A decrease in density of 1% could potentially translate into a nearly 2% lower risk of developing breast cancer [27]. ***Effects of HT on Breast Density:*** Data from epidemiological studies demonstrated that increase in olive oil intake was associated with decrease in breast densities[30]. A series of 2,000 women who had undergone routine regular mammography and who had detailed information regarding dietary and lifestyle available were evaluated. The authors reported that women in the highest tertile of olive oil consumption (>30.5 g/day) were associated with 30% reduction in breast density[30].

2.1 Methodology

The mammographic density will be classified by two different methods. Firstly, according to the American College of Radiology's Breast Imaging―Reporting and Data System (BIRADS), and secondly, by using the Cumulus software package developed by the Ontario Cancer Institute to assess percent density as a continuous measure (0-100% scale). The mammograms will be read for research purposes only. They will be read for clinical purposes at the time of the mammogram. The mammograms will be independently reviewed by two radiologists specializing in breast imaging. Quantitative classification of mammographic parenchyma will be based on radiological assessment after digitizing mammograms and measuring percentage density[28, 31].

2.2 Risk Assessment

- Breast Risk Calculation using the modified Gail risk score [32, 33]. This model is an interactive tool designed to estimate a woman's risk of developing invasive breast cancer in her lifetime and the next 5 years.
  - http://www.cancer.gov/bcrisktool/
  - This model can only be used if there is no known diagnosis of DCIS or LCIS
- A patient with a diagnosis of lobular carcinoma in situ (LCIS) or atypical lobular or atypical ductal hyperplasia. Germline mutations of BRCA 1 or BRCA 2 genes. Germline mutations in the PTEN (cowden syndrome) and TP53(Li-Fraumeni syndrome)
- History of unilateral breast cancer 5 years ago
- At least 10% probability of carrying BRCA mutation

3 Objectives and endpoints

Primary

- To conduct a pilot breast cancer prevention study of hydroxytyrosol in women at increased risk of breast cancer.
- To assess whether mammographic density is reduced in pre or post menopausal women at high risk of breast cancer taking hydroxytyrosol for 1 year compared with baseline.

Secondary

- To assess the toxicity of hydroxytyrosol
- To evaluate FACT ES score baseline, 6 months and 1 year.
-

Exploratory objectives (Mandatory biopsy)

- To assess whether proliferation (Ki67 staining, Cdkn2a tumor suppressor, cyclin D1), is reduced in women at high risk of breast cancer after taking hydroxytyrsol for 1 year compared with baseline.
- To explore the difference in the expression of other biomarkers of apoptosis (Fas receptor, cleaved caspase-3, caspase-8, cytochrome c), enzymes related to oxidative stress (Fas receptor, cleaved caspase-3, caspase-8, cytochrome c), DNA damage by immunohistochemistry, Wnt signaling(LRP6, β-catenin, Sfrp proteins 1 to 5, DKK1, c-jun, c-myc) and stem cell pathway before and after drug therapy.
- To collect and bank breast tissue from women at high risk of breast cancer prior to and after treatment with hydroxytyrosol for future biomarker analysis.

Exploratory endpoint (MRI Breast Imaging)

- To assess breast MRI density pretreatment and 12 months post hydroxytyrosol therapy in subjects with available breast MRIs.

4 Study design

4.1 Description of study design

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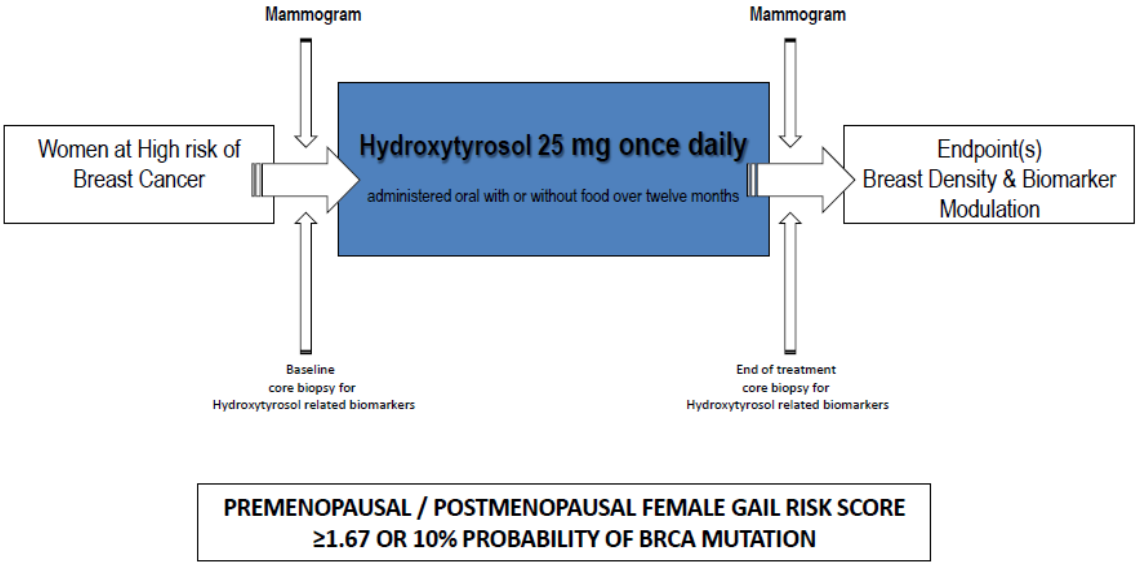

One hundred (100) pre and post-menopausal women at high risk of breast cancer who have declined tamoxifen, raloxifene or aromatase inhibitor (standard of care) will receive 25mg HT. Eligible women will have a 5 yr risk by Gail Model ≥ 1.7, or 10% probability of BRCA mutation by BRCAPRO or similar model, or have a previous breast biopsy showing LCIS, atypical lobular or ductal hyperplasia, or have a known germline mutation in *BRCA1* or *BRCA2*. Patients with unilateral breast cancer 5 or more years prior to registration are also allowed. The main exclusion criteria include women who are pregnant or breast feeding, with diagnosis of any malignant disease, or with inability to take oral medication. The women will undergo a mammogram as well as a biopsy of normal breast tissue before and after taking study drug. This biopsy will be directed to the upper outer quadrant, which is typically a dense region. The primary endpoint of this study is change in mammographic density after treatment with HT. The Secondary endpoint will assess toxicity, apoptosis (Fas receptor, cleaved caspase-3, caspase-8, cytochrome c) and cell proliferation (Ki 67 staining, Cdkn2a tumor suppressor, cyclin D1), enzymes related to oxidative stress (ROS and related enzymes), DNA damage by immunohistochemistry, stem cell markers (CD44, CD24, and ALDH), and affectation of Wnt signaling (LRP6, β-catenin, Sfrp proteins 1 to 5, DKK1, c-jun, c-myc) before and after drug therapy. If patients undergo a breast MRI before and/or after the completion of the drug, the images will be collected. The study drug will be supplied by PLThomas & Co.. The patients will have a study visit at3, 6 and 12 months. The study visit will include a general history and physical exam, an assessment for safety and concomitant medications and pill

count. After completion of therapy, the patient will undergo a mammogram as well as a second breast biopsy.

5 Study Population

Each of the criteria in the following section must be met in order for a participant to be considered eligible for registration.

Study Duration:

The duration of patient participation in the study treatment will be a total of 12 months, which is counted from the start of treatment. Study Follow-up for all patients will include medical history update (30 days after the last dose of the study drug)

Safety Criteria:

All participants will be assessed by a physician for pre-existing medical conditions and baseline physical abnormalities prior to the initiation of investigational therapy. Patients presenting with any medical history, physical exam, or laboratory abnormality that, in the in the opinion of the treating physician, would put the subject's safety at risk will be excluded. Baseline signs and symptoms are to be recorded and followed throughout the trial. These will be monitored throughout the study and recorded if they increase in severity or frequency during treatment or within the follow up period. Participants will be assessed for adverse events by a physician or designated midlevel provider while the subject is on study. Vital signs including blood pressure, heart rate and temperature should be performed at each physical exam. Assessments may be performed more frequently if clinically indicated. In addition, hematology and serum chemistry profiles will be drawn prior to the initiation of treatment to determine whether the study drug combination affects hematologic values, electrolytes or liver function tests. Laboratory assessments will be performed more frequently if clinically indicated. This clinical and laboratory data will be used to determine whether these women in the study with Hydroxytyrosol have any symptoms or side effects associated with the study medications. Subjects will be followed for adverse events for a period of 30 days after the completion of investigational therapy. Patients with abnormal laboratory or clinical findings that are believed to be treatment related will be followed until the condition resolves or stabilizes, or until the laboratory values are no longer considered clinically significant.

CTC Version 4.0 will be used to grade toxicities. Laboratory tests may be done more frequently if medically indicated. If a participant develops any grade 3 or grade 4 toxicity possibly, probably or definitely related to the study drug, the study drug will be discontinued. If the participant develops grade 1 or 2 toxicities that are felt to be possibly, probably or definitely related to the study drug, the participant may go on a drug holiday (for no longer than 12 consecutive weeks) and restart the drug at the same dose. In each of these cases, the follow-up mammograms, breast biopsies will still be collected as per protocol one year after the initial start date of the intervention.

If CTC Grade 3 or 4 hematologic toxicity is seen, CBC + differential + platelets should be repeated weekly until recovery.

5.1 Inclusion and Exclusion criteria

Patients must have baseline evaluations performed prior to the first dose of study drug and must meet all inclusion and exclusion criteria. Results of all baseline evaluations, assuring that all inclusion and exclusion criteria have been satisfied, must be reviewed by the Principal Investigator or his/her designee

prior to enrollment of that patient. In addition, the patient must be thoroughly informed about all aspects of the study, including the study visit schedule and required evaluations and all regulatory requirements for informed consent. The written informed consent must be obtained from the patient prior to enrollment. The following criteria apply to all patients enrolled onto the study unless otherwise specified.

1. Participants must have ≥18 years of age.
2. Participants must have an elevated risk of breast cancer as defined by at least one of the following categories and have declined tamoxifen, raloxifene and/or aromatase inhibitor therapy:
   a. Diagnosis of LCIS, atypical ductal or lobular hyperplasia.
   b. A known deleterious mutation in *BRCA1*, *BRCA2, PTEN* or *TP53*. (Note: The participant must be a documented carrier to meet this criterion. If there is a known mutation in a hereditary breast cancer susceptibility gene in a participant's family member, the participant herself must have undergone genetic testing as per NCCN clinical guidelines to be eligible per this criterion.) Known mutation in PALB2 or CHEK2 del 100C or any germline mutation.
   c. Modified Gail/CARE model risk at 5 years ≥ 1.67%. (Note: Risk models are to be used only if there is no known previous diagnosis of resected DCIS or LCIS and there is no known deleterious mutation in *BRCA1, BRCA2, PTEN* or *TP53*).
   d. 10% or more probability of BRCA mutation by BRCAPRO or similar model
3. Participants must have at least one breast available for imaging and biopsy. A previously irradiated breast (i.e., for resected DCIS) is not evaluable for breast imaging or biopsy.
   a. _____ Participants must not have a recent diagnosis of invasive breast cancer, however, women with breast cancer who have been disease-free for more than 5 years are eligible. They will undergo standard follow-up evaluations appropriate to their recurrent cancer risk.
4. Participants must allow submission of core needle breast material (obtained per Section 7.3) for future use
5. Participants must have a baseline mammogram performed within 90 days prior to study entry, done on a digital mammography machine that shows either normal or benign findings.
   Participants with mammograms that are reported as suspicious for malignancy are eligible as long as the biopsy is negative.
6. Participants must have baseline mammographic density > 10% based upon the classification system (2 = 11-50%, "scattered fibroglandular densities"; 3 = 51-75%, "heterogeneously dense"; 4 = >75%, "extremely dense"). Women with a baseline mammographic density of ≤ 10% (1 = ≤10%, breasts are almost entirely fat) will not be eligible. Women with a BIRADs score of 4 will be considered eligible as long as the biopsy is benign.
7. Prior tamoxifen, raloxifene or aromatase inhibitor use is allowed provided treatment is completed at least 1 year prior to registration.
8. Participants must not have bilateral breast implants, but prior breast reduction surgery is allowed. (Breast implants are not allowed as they affect density measurements and because of the risk of rupturing the implant with biopsy).
9. Participants must have a ECOG Performance Status of 0 – 1 (see Section 11).
10. Prior anticoagulant therapy use is allowed provided therapy is discontinued at least 7 days prior to the breast biopsy in order to reduce the risk of bleeding. For subjects who have taken an anticoagulation within the past 7 days, INR (International Normalized Ratio) must be ≤ 1.5 x institutional upper limit of normal and Prothrombin Time and Partial Thromboplastin Time ≤ IULN prior to the breast biopsy.
11. No prior malignancy is allowed within the past five years except for the following: adequately treated skin cancer and *in situ* cervical cancer .

12. Participant must not be pregnant or nursing and must agree to use effective contraception. Hormone-based birth control (pills, patches or shots) are allowed, but switching birth control methods is discouraged while on-study as hormonal changes can affect mammographic density. Hormone replacement therapy is not allowed for post-menopausal female.
13. Individuals must not participate in any other clinical trial for the treatment or prevention of cancer unless they are no longer receiving the intervention and are in the follow-up phase only. Participants must also agree not to join such a trial while participating in this study.
14. All participants must be informed of the investigational nature of this study and must sign and give written informed consent in accordance with institutional and federal guidelines.

Exclusion Criteria

1. No prior malignancy within the past five years except for the following: adequately treated basal cell or squamous cell skin cancer and *in situ* cervical cancer. .
2. Prior Tamoxifen or Raloxifene or aromatase inhibitor or hormone replacement therapy use in the past 1 year

6 Treatment

6.1 Study treatment

Hydroxytyrosol will be administered at 25 mg oral daily.

All dosages prescribed and dispensed to the patient and all dose changes during the study must be recorded.

Each bottle will contain 90 capsules. Each capsule will contain 250 mg of Hytolive powder (containing 25 mg of hydroxytyrosol). Medication labels will comply with US legal requirements and be printed in English. They will supply no information about the patient. The storage conditions for Hydrotyrosol will be described on the medication label.

Hydrotyrosol is supplied by PLThomas & Co, Inc. Hydrotyrosol is formulated as capsules for oral administration of 25mg, Capsules

6.1.1 Dosing regimen

The investigator should promote compliance by instructing the patient to take the study drug exactly as prescribed and by stating that compliance is necessary for the patient's safety and the validity of the study. The patient should be instructed to contact the investigator if he/she is unable for any reason to take the study drug as prescribed.

Hydroxytyrosol should be administered orally once daily at the same time every day, either consistently with or consistently without food

Capsules

The capsules should be swallowed whole with a glass of water and should not be chewed or crushed.

If vomiting occurs, no attempt should be made to replace the vomited dose. Patients should be instructed that if they miss a dose on one day, they must not take any extra dose the next day, but instead to immediately contact the study center as soon as possible to ask for advice.

6.2 Dose modifications

6.2.1 Dose modification and dose delay

If the participant develops grade 1 or 2 toxicities that are felt to be possibly, probably or definitely related to the study drug, the participant may go on a drug holiday (for no longer than 12 consecutive weeks) and restart the drug at the same dose. In each of these cases, the follow-up mammograms, breast biopsies will still be collected as per protocol one year after the initial start date of the intervention.

6.2.2 Visits

Before the drug start, the participants will have a general physical exam including a clinical breast exam, (with consent), height, weight, medical history, abdominal circumference, blood tests (CBC, CMP, fasting lipid panel), and a bilateral mammogram (if one not available within 90 days of registration). Breast MRI images will be collected and reviewed if available. If the mammogram is read as normal or benign , participants will undergo a non-directed core needle biopsy within 30 days of signing consent. For subjects who have taken an anticoagulation within the past 7 days, INR (International Normalized Ratio) must be ≤ 1.5 x institutional upper limit of normal and Prothrombin Time and Partial Thromboplastin Time ≤ IULN prior to the breast biopsy. If the patient has had a bone density (within the past 1 year) than it will be collected. Participants will then receive oral hydroxytyrosol for 1 year. A six month supply of study drug will be dispensed at Day 1 and another 6 months sample on month 6 visit. Participants will complete the self-administered FACT ES score at baseline, 6 months as well as at 12 months (Form ES).

6.2.2.1 Follow Up(s)

For follow-up visits, the participant will be seen at the study site at Months 3, 6 and 12. Follow-up assessments are based from the date of treatment start. Clinic visit should occur on Days 90, 180 and 360 (+/- 14 days for each assessment) to allow flexibility in scheduling. During these visits, counts of capsules will be recorded on the Treatment Form
and in the participant's medical record. At month 13, the patient will receive a phone call for toxicity check.
Reported side-effects will also be assessed during these visits. Physical and clinical breast exam will be done at Months 3, 6 and 12. In addition, blood (CBC, CMP, fasting lipid panel) will be collected to monitor for toxicity at baseline, 3,6 and 12 months (see Study Calendar). Pill dispensing will be done at baseline and the Month 6 visit.

6.2.2.2 Drug Completion Visit (Month 12; Day 360 +/- 14 days)

After the 12 month intervention, all participants will have a complete physical exam including clinical breast exam. Once the participant completes the study drug, she should have the bilateral mammogram and biopsy scheduled within 4 weeks of stopping the intervention. If a subject has a breast MRI, the images will be collected. Participants will complete the self-administered Patient Questionnaire (FACT ES) at the completion of the treatment.

6.2.2.3 Compliance

The investigator/designee will count remaining unused hydroxytrosol at each visit to assess compliance. A study medication log is provided in the CRF to capture subject compliance. If the subject forgets to return unused hydroxytrosol at a scheduled visit, compliance will be confirmed by direct questioning of the subject. Subjects should be instructed to record any missed doses of hydroxytrosol.

Compliance with hydroxytrosol will be calculated by the ratio of number of pills actually taken to the number of pills that should have been taken. Compliance will be defined as a ratio ≥ 0.75. Subjects with ratios between 1.00 and 0.75 will be allowed to continue on study. The importance of compliance with study medication should be reinforced to subjects with a ratio of less than 1.00. Subjects who have ratios < 0.75 will be taken off study for non-compliance.

Compliance with hydroxytrosol will be evaluated by a physical pill count. In the event that the patient forgets to bring unused drug/empty drug bottles to the study visit, patients will be queried by study staff regarding compliance.

6.2.2.4 Post Intervention Follow Up Phone Call (Month 13; Day 390 +/- 7 days):

Participants will be called for assessment and/or resolution of adverse events.

6.2.2.5 Biopsy Procedures

Participants will undergo core needle biopsy of normal breast tissue and the entire specimen will be submitted in total to research. The patient will not receive any results of the biopsy. Which breast to sample for biopsy will be at the discretion of the treating physician. This biopsy will not be directed at a particular lesion, but instead will be directed to the upper outer quadrant of the breast, which is typically a dense region of the breast. Core needle biopsy of the breast will be performed using a 14-gauge needle. After providing local anesthesia, a small skin incision is made in the breast and the core needle inserted. Up to four core biopsies are taken through this single incision, and after completing the biopsies, the wound is steri-stripped, dressed, and ice is placed on the wound to minimize bruising and scarring. The participant is encouraged to keep ice on the biopsy site for up to 2 hours. Part of the breast biopsy specimen will be saved as frozen tissue and other part will be placed in fixative for H & E and immunohistochemical staining for biomarkers. Potential complications include bleeding, pain, hematoma formation and bruising, or infection. The first biopsy will be before beginning hydroxytyrosol and the second biopsy will be done after treatment with hydroxytyrosol for one year. There will be no attempt to biopsy the exact same area of the breast or the same on the second breast biopsy. Instead, the upper outer quadrant of the breast will again be sampled.

Breast tissue can be collected from a scheduled biopsy or surgery and used in place of the breast biopsy.

Rationale for Mandatory Biopsy before and after the treatment with hydroxytyrosol.

Identifying risk biomarkers in benign breast tissue may provide direct measure of the effect of treatment on target tissue beyond any information imaging can provide. Ki-67 expression in benign breast tissue has been included into many early phase chemoprevention trials[34,35]. Unfortunately there are many limitations of using Ki67 expression in benign tissue (due to very low baseline Ki67 in these patients) and other tissue biomarkers are necessary. Therefore biopsy including markers for apoptosis, stem cell as well as Wnt Pathway (primary action of hydroxytyrosol) is proposed. Ki-67 expression does not have correlation with mammographic density therefore it is possible for the patient to have tissue changes but not able to detect it in the mammogram[36].

The samples will be stored at:
Houston Methodist Research Institute Biorepository Core
6565 Fannin St. Ste. F774 (Foundren Building)
Houston Texas, 77030

6.2.2.6 FACT – ES survey

The current standard of care in patients with increased risk of developing breast cancer is anti-estrogen therapy. The FACT-ES score has been validated in patients receiving endocrine therapy. We are using

the FACT-ES score at baseline, 6 months and 1 year so that we can historically compare the side effects in patients on hydroxytyrosol to known endocrine side effects.

6.2.2.7 Breast MRI

Breast MRI images will be collected if available and are not a required test for study participation. The images available will be used to calculate MRI breast density. The axial view T1-weighted images without fat suppression will be used for the analysis of breast density in this study. The images will be acquired using a 2D turbo spin-echo pulse sequence with TR=800 ms, TE=8.6 ms, flip angle=90°, matrix size=480x480, FOV=31–38 cm, and slice thickness=2 mm. The breast and fibroglandular tissue segmentation will be performed using a modified published method. [31-33] Before the segmentation, the operator will view the whole axial T1W images dataset and determined the superior and inferior boundaries of the breast (the beginning and ending slices) by comparing the thickness of breast fat with the body fat. The breast segmentation procedures consisted of: 1) Perform an initial horizontal line cut along the posterior margin of each individual subject's sternum to exclude thoracic region. 2) Apply Fuzzy-C-Means (FCM) clustering and b-spline curve fitting to obtain the breast-chest boundary. 3) A bias field correction method based on nonparametric nonuniformity normalization (N3) and adaptive FCM algorithm [33] will be used to remove the strong intensity non-uniformity for segmentation of fibroglandular tissue and fatty tissue. 4) Apply dynamic searching to exclude the skin along the breast boundary. 5) The standard FCM algorithm is applied to classify all pixels on the image. The default setting is to use a total of 6 clusters, 3 for fibroglandular tissue and 3 for fatty tissues. After completing the segmentation processes in all image slices, the quantitative breast volume, fibroglandular tissue volume, and the percent density (calculated as the ratio of the fibroglandular tissue volume over the breast volume x100%), will be calculated.

6.2.2.8 Criteria for Removal from Protocol Treatment

a. Evidence of any cancer at any time, including DCIS. If the pathologic review of the baseline core needle breast biopsy confirms invasive breast cancer, the participant must be removed from protocol treatment.
b. Unacceptable toxicity (as defined in Section 8.0).
c. A participant who becomes pregnant while on treatment will be removed from protocol treatment since hormonal changes affect mammographic density.
d. Delay of study intervention > 12 consecutive weeks due to any reason.
e. Completion of twelve months of study intervention.
f. The participant may withdraw from the study at any time for any reason.
g. All reasons for discontinuation of the intervention must be documented in the Off Treatment Notice – Prevention Studies.
8. All participants will be followed for 13 months after registration. After 13 months, no further follow-up is required.
9. Non-compliance with study drug.

6.2.2.9 Withdrawal from Protocol Treatment

Follow-up visits will continue even if the participant goes off study intervention early unless there is a withdrawal of consent.

7 Visit schedule and assessments

7.1 Study flow and visit schedule

Table 7-1 Visit evaluation schedule

Required Studies	Prestudy Evaluation	Day 1	Month 1-2 (Day 30 to 60)	Month 3 (day 90)[1]	Month 6 (day 180)[1]	Month 9 (day 270)[1]	Month 12 (day 360)[1]	Month 13 (day 390)[1]
Clinic Visit								
Breast Cancer Risk Assessment[2]	X							
Baseline and Pre-existing conditions	X							
History and Physical Exam[13]	X			X	X		X	
Height and Weight	X						X	
Vital Signs	X			X	X		X	
Performance Status	X						X	
Toxicity Notation[3]				X	X		X	X[12]
Review of Intake Calendar[4]				X	X		X	
Laboratory								
Serum liver function tests	X			X	X		X	
Serum lipid panel (fasting)	X			X	X		X	
PT/PTT/INR[5]	X						X	
FSH (if necessary)[6]								
CBC and CMP	X			X	X		X	
Serum Pregnancy[14]	X							
Radiographs								
Bilateral Mammogram[7]	X						X	
Bilateral Breast MRI image collection[7]	X						X	
Bone Density[8]	X						X[8]	
Procedures								

Random core biopsy[9]	X						X	
Intervention								
Dispense study drug[10]		X			X			
Count Pills				X	X		X	
Questionnaire[11]	X				X		X	

1. Followup visits can occur (+/- 14 days for each assessment) to allow flexibility in scheduling. Follow-up visits will continue even if the participant goes off study intervention early unless there is a withdrawal of consent.
2. Risk assessment using Gail risk model if no known history of DCIS or LCIS and no deleterious mutation in BRCA 1 or 2, PTEN or P53
3. On months 3, 6, 12 (Days 90, 180, 360 (+/- 14 days for each assessment)) adverse events will be noted
4. On months 3, 6, and 12 (90, 180, 360 +/- 14 days for each assessment), participants will be seen at the study sites to review the intake calendar which will document drug compliance and date of last menses.
5. PT/PTT/INR required if the patient has been on anticoagulation therapy within 7 days prior to breast core biopsy.
6. FSH only to be performed if menopausal status needs to be determined (e.g. in patient with hysterectomy with at least one ovary intact – see section 7.2.2)
7. Mammogram within 90 days of registration. Imaging to be done prior to breast biopsy. Bilateral Breast MRI images to be collected if conducted within 90 days of registration at baseline End of treatment Breast MRI images to be collected if available.
8. This test information will be collected if available. Bone density not to be ordered specifically for the study..
9. The random core needle biopsy is mandatory for study participation. All new patients on the study will undergo biopsy before and after the study drug. No attempt will be made to have the patients already on the study to undergo mandatory biopsy. All imaging is to be done prior to breast biopsy. If a patient is scheduled for a breast biopsy or surgery, tissue can be collected at that time.
10. A six month supply of the study drug will be dispensed at Day 1 visit and at 6 months. Compliance to be verified via pill count and pill diary.
11. The Fact ES questionnaire will be given at baseline, 6 months and at 12 months
12. Phone call to assess adverse events.
13. Physical exam to include abdominal circumference at baseline, 3, 6 and 12 months
14. Serum pregnancy test if premenopausal and has not had tubal ligation.

7.2 Assessment types

Followup visits can occur (+/- 14 days for each assessment) to allow flexibility in scheduling. Follow-up visits will continue even if the participant goes off study intervention early. Risk assessment using Gail risk model if no known history of DCIS or LCIS and no deleterious mutation in BRCA 1 or 2, PTEN or P53. On months 3, 6, and 12 and 13 (Days 90, 180, 360 and 390 (+/- 14 days for each assessment)) adverse events will be noted. On months 3, 6, and 12 (90, 180, 360 +/- 14 days for each assessment), participants will be seen at the study sites to review the intake calendar which will document drug compliance and date of last menses. PT/PTT/INR required if the patient has been on anticoagulation during the past 7 days. FSH only to be performed if menopausal status needs to be determined (e.g. in patient with hysterectomy with at least one ovary intact). Mammogram within 90 days of registration. Bilateral Breast MRI will not be performed specifically for this study but if the images available within the past 90 days, they will be collected. Bone density not to be ordered specifically for the study and if not available does not exclude the patient from participating. The random core needle biopsy is mandatory for study participation. A six month supply of the study drug will be dispensed at Day 1 and at 6 months. The Fact ES questionnaire will be given at baseline, 6 months and at 12 months.

7.2.1 Pregnancy and assessments of fertility

Pregnancy testing is required at screening for premenopausal patients that have not had tubal ligation or whenever pregnancy is suspected. Serum pregnancy testing should be performed at screening

7.2.2 Determination of Menopausal Status

The following criteria will be used to define *postmenopausal:*

- Age 56 or older with no spontaneous menses for at least 12 months prior to study entry; **or**
- Age 55 or younger with no spontaneous menses for at least 12 months prior to study entry (e.g., spontaneous or secondary to hysterectomy) **and** with a documented estradiol level in the postmenopausal range according to local institutional/laboratory standard; **or**
- Documented bilateral oophorectomy.

Women failing to meet one of these criteria will be classified as *pre-menopausal*.

7.2.2 Drug levels and pharmacokinetic assessments N/A

8 Safety monitoring and reporting

Information about all adverse events, whether volunteered by the subject, discovered by investigator questioning, or detected through physical examination, laboratory test or other means, will be collected and recorded and followed as appropriate.

8.1 Adverse events

8.1.1 Definitions and reporting

Adverse events that begin or worsen after informed consent should be recorded in the Adverse Events CRF. Conditions that were already present at the time of informed consent should be recorded in the Medical History page of the patient's CRF. Adverse event monitoring should be continued for at least 30 days (or 5 half-lives, whichever is longer) following the last dose of study treatment. Adverse events

(including lab abnormalities that constitute AEs) should be described using a diagnosis whenever possible, rather than individual underlying signs and symptoms. When a clear diagnosis cannot be identified, each sign or symptom should be reported as a separate Adverse Event.

The occurrence of adverse events should be sought by non-directive questioning of the patient at each visit during the study. Adverse events also may be detected when they are volunteered by the patient during or between visits or through physical examination, laboratory test, or other assessments. As far as possible, each adverse event should be evaluated to determine:

1. The severity grade (CTCAE Grade 1-4)
2. Its duration (Start and end dates or if continuing at the Safety Follow-up Visit)
3. Its relationship to the study treatment (Reasonable possibility that AE is related: No, Yes)
4. Action taken with respect to study or investigational treatment (none, dose adjusted, temporarily interrupted, permanently discontinued, hospitalized, unknown, not applicable)
5. Whether medication or therapy was given (no concomitant medication/non-drug therapy, concomitant medication/non-drug therapy)
6. Outcome (not recovered/not resolved, recovered/resolved, recovering/resolving, recovered/resolved with sequalae, fatal, unknown)
7. Whether it is serious, where a serious adverse event (SAE) is defined as in Section 8.2.1

All adverse events should be treated appropriately. Such treatment may include changes in study drug treatment including possible interruption or discontinuation, starting or stopping concomitant treatments, changes in the frequency or nature of assessments, hospitalization, or any other medically required intervention. Once an adverse event is detected, it should be followed until its resolution, and assessment should be made at each visit (or more frequently, if necessary) of any changes in severity, the suspected relationship to the study drug, the interventions required to treat it, and the outcome.

Information about common side effects already known about the investigational drug can be found in the [Investigators' Brochure]. This information should be included in the patient informed consent and should be discussed with the patient during the study as needed.

Adverse event monitoring should be continued for at least 30 days following the last dose of study treatment

8.1.2 Laboratory test abnormalities

Laboratory abnormalities that constitute an Adverse event in their own right (are considered clinically significant, induce clinical signs or symptoms, require concomitant therapy or require changes in study treatment), should be recorded on the Adverse Events CRF. Whenever possible, a diagnosis, rather than a symptom should be provided (e.g. anemia instead of low hemoglobin). Laboratory abnormalities that meet the criteria for Adverse Events should be followed until they have returned to normal or an adequate explanation of the abnormality is found. When an abnormal laboratory or test result corresponds to a sign/symptom of an already reported adverse event, it is not necessary to separately record the lab/test result as an additional event.

Laboratory abnormalities, that do not meet the definition of an adverse event, should not be reported as adverse events. A Grade 3 or 4 event (severe) as per CTCAE does not automatically indicate a SAE unless it meets the definition of serious as defined below and/or as per investigator's discretion. A dose hold or medication for the lab abnormality may be required by the protocol and is still, by definition, an adverse event.

8.2 Serious Adverse Events

8.2.1 Definitions

A serious adverse event is an undesirable sign, symptom or medical condition which:

- is fatal or life-threatening
- results in persistent or significant disability/incapacity
- constitutes a congenital anomaly/birth defect
- requires inpatient hospitalization or prolongation of existing hospitalization, unless hospitalization is for:
  - routine treatment or monitoring of the studied indication, not associated with any deterioration in condition (specify what this includes)
  - elective or pre-planned treatment for a pre-existing condition that is unrelated to the indication under study and has not worsened since the start of study drug
  - treatment on an emergency outpatient basis for an event not fulfilling any of the definitions of a SAE given above and not resulting in hospital admission
  - social reasons and respite care in the absence of any deterioration in the patient's general condition
- is medically significant, i.e., defined as an event that jeopardizes the patient or may require medical or surgical intervention to prevent one of the outcomes listed above

8.2.2 Reporting

The principal investigator has the obligation to report all serious adverse events to the FDA, and institutional IRB.

All events reported to the FDA by the investigator are to be filed utilizing the Form FDA 3500A (MedWatch Form).

To ensure patient safety, every SAE, regardless of suspected causality, occurring

- after the patient has provided informed consent and until at least 30 days after the patient has stopped study treatment/participation
- after protocol-specified procedures begin (e.g., placebo run-in, washout period, double-blind treatment, etc.) and 30 days after the patient has stopped study treatment
- after the start of any period in which the study protocol interferes with the standard medical treatment given to a patient (e.g., treatment withdrawal during washout period, change in treatment to a fixed dose of concomitant medication) and until 30 days after the patient has stopped study treatment

must be reported to The Methodist hospital research Institute and The Methodist hospital Cancer Center within 24 hours of learning of its occurrence **(fax: 713-793-1642)**. This includes serious, related, labeled (expected) and serious, related, unlabeled (unexpected) adverse experiences. All deaths during treatment or within 30 days following completion of active protocol therapy must be reported within 5 working days.

Any SAEs experienced after this 30 days period should only be reported to TMHS and TMHCC if the investigator suspects a causal relationship to the study drug. Recurrent episodes, complications, or progression of the initial SAE must be reported as follow-up to the original episode within 24 hours of the investigator receiving the follow-up information. A SAE occurring at a different time interval or otherwise considered completely unrelated to a previously reported one should be reported separately as a new event. The end date of the first event must be provided.

The original copy of the SAE Report and the fax confirmation sheet must be kept within the Trial Master File at the study site.

Follow-up information is sent to the same fax number as the original SAE Report Form was sent, using a new fax cover sheet, stating that this is a follow-up to the previously reported SAE, and giving the date of the original report. Each re-occurrence, complication, or progression of the original event should be

reported as a follow-up to that event regardless of when it occurs. The follow-up information should describe whether the event has resolved or continues, if and how it was treated, whether the blind was broken or not (if applicable), and whether the patient continued or withdrew from study participation.

If the SAE is not previously documented in the Hydroxityrosol Investigator Brochure or Package Insert (new occurrence) and is thought to be related to the study drug, may urgently require further information from the investigator for Health Authority reporting. TMHCC and TMHRI may need to issue an Investigator Notification (IN), to inform all investigators involved in any study with the same drug that this SAE has been reported. Suspected Unexpected Serious Adverse Reactions (SUSARs) will be collected and reported to the competent authorities and relevant ethics committees in accordance with Directive 2001/20/EC or as per national regulatory requirements in participating countries.

For Comparator Drugs/Secondary Suspects (Concomitant Medications), all serious adverse experiences will be forwarded to the comapartor drug company by the investigator.

9 Statistical methods

Study Design and Sample Size. This is a single-arm, pilot breast cancer prevention study of hydroxytyrosol in women at increased risk of breast cancer. Our primary study endpoint will be defined as proportion of women who exhibit a 10% absolute reduction in mammographic density at one-year of follow-up (Cuzick JNCI 2011). For each of the post and pre-menopausal group of women, we will assume under the null hypothesis that 25% of women will exhibit a response (at least a 25% reduction from baseline) versus an alternative hypothesis equal to 45% based on data presented from Cuzick (JNCI 2011). Under these assumptions, a sample of 50 pre-menopausal and 50 post-menopausal women will provide 85% power based on a two-sided test for a single proportion with 5% significance level for each group of women.

Statistical Analysis. Demographics and pre-treatment characteristics will be summarized for 50 premenopausal & 50 postmenopausal women using descriptive statistics. Mammographic density levels at baseline and follow-up time-points as well as categorized levels based on BIRAD criteria will also be summarized using means, standard deviations and proportions. Change from baseline levels of breast density will be analyzed using paired tests while the proportion of women who exhibit at least a 10% absolute reduction from baseline will be calculated along with 95% binomial confidence intervals for each group of women.

Correlative endpoints including Ki-67, apoptosis, Wnt signaling and stem cell pathway markers will likewise be summarized descriptively at each time point of follow-up. Correlations between these markers and with mammographic density levels will be assessed using Spearman or Pearson's correlation coefficients. Change from baseline levels will be analyzed using paired t-test or Wilcoxon-signed rank test.

Multivariate analysis to adjust for demographic/clinical characteristics, specifically age or menopausal status (pre, peri, and post) will likewise be considered using logistic regression for analyzing proportion of women with response or using linear regression models for analysis of quantitative levels of mammographic density. Given the pilot nature of this study, these multivariate analyses will be considered exploratory.

Expected outcome: We expect a decrease in apoptosis, cell proliferation, in post-treatment breast tissue in comparison to pre-treatment. Our previous data reveal an inhibition of Wnt signaling by HT, thus we expect a change in this signaling in this prenvention study.

Potential pitfalls, and resolutions: If we do not have enough breast tissue for all determinations, it will be difficult to measure mRNA levels. In such a scenario, we can determine the expression of Sfrp proteins only by multiplex nucleic acid *in situ* hybridization technology (RNAscope) in paraffin embedded tissue slides.

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11 Appendices

Appendix A: ECOG Performance Status Criteria

ECOG Performance Status Scale	
Grade	**Descriptions**
0	Normal activity. Fully active, able to carry on all pre-disease performance without restriction.
1	Symptoms, but ambulatory. Restricted in physically strenuous activity, but ambulatory and able to carry out work of a light or sedentary nature (e.g., light housework, office work).
2	In bed < 50% of the time. Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	In bed > 50% of the time. Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	100% bedridden. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Dead

Appendix B: New York Heart Association (NYHA) Classifications

Class	Description
I	Patients with cardiac disease but without resulting limitations of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, dyspnea, or anginal pain.
II	Patients with cardiac disease resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnea, or anginal pain.
III	Patients with cardiac disease resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary physical activity causes fatigue, palpitation, dyspnea, or anginal pain.
IV	Patients with cardiac disease resulting in inability to carry on physical activity without discomfort. Symptoms of cardiac insufficiency or of the anginal syndrome may be present event at rest. If any physical activity is undertaken, discomfort is increased.

This table is an excerpt from the Oxford Textbook of Medicine, 2nd ed. Oxford; New York: Oxford University Press, 1987, p. 2228.

Appendix C: CTCAE Files

Version 4.0 (dated June-14-2010)

NCI Common Terminology Criteria for Adverse Events (CTCAE) v.4 data files and related documents are published here. The most current release files appear in this directory:

Files: Booklet	Content
CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf	Most recent release of core terminology: PDF document, traditional small booklet format.

http://evs.nci.nih.gov/ftp1/CTCAE/About.html

Appendix D: Trial Logistics

No. of Centers:

Key Milestones:

FPFV / First Dose: With documented ERligibility for a high risk Study.

LPLV / Last Subject completed:

Database Lock:

Final Report: -

Sponsoring Department: TMHCC _____TMHRI _____TMHS_____

Prepared by: _____ Date _____