PROTOCOL FACE SHEET

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SIGNATURE PAGE

By signing this page, it is agreed that you will conduct the study as dictated by this protocol.

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Site Principal Investigator Printed Name	Site Co-Principal Investigator Printed Name
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Date of Site Principal Investigator Signature	Date of Site Co-Principal Investigator Signature

Please sign and return this signature page to the Protocol Chair before initiating study.

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List of abbreviations

AE	Adverse Event
AI	Aromatase Inhibitor
ALT	alanine aminotransferase
AST	aspartate aminotransferase
b.i.d.	bis in diem/twice a day
BMD	Bone mineral density
CEE	Conjugated equine estrogen
CI	Confidence interval
CRF	Case Report/Record Form
CS&E	Clinical Safety and Epidemiology
CR	Clinical Research
CRO	Contract Research Organization
DCIS	Ductal carcinoma in situ
ECG	Electrocardiogram
ER	Estrogen receptor
HR	Hazard ratio
IEC	Independent Ethics Committee
i.v.	intravenous(ly)
IRB	Institutional Review Board
MPA	Medroxyprogesterone acetate
o.d.	omnia die/once a day
p.o.	per os/by mouth/orally
REB	Research Ethics Board
RPFNA	Random periareolar fine needle aspiration
SAE	Serious Adverse Event
SHBG	Sex hormone binding globulin
SOP	Standard Operating Procedure
WHI	Women's Health Initiative
WHO	World Health Organization

SCHEMA

Prevention Visit

Risk assessment of postmenopausal potential study subjects currently using hormones to control menopause symptoms.

Gail 5-year risk ≥1.7% and/or a relative risk of ≥3X that for the average woman of the same age, as calculated by the NCI Breast Cancer Risk Assessment Tool;
or a 10-year relative risk of $\geq$ 3X that for the population, as calculated by the IBIS Breast Cancer Risk Evaluation Tool;
or BRCA1/2 mutation carrier; or prior ADH, DCIS, LCIS,

↓

Qualifying RPFNA visit

Bone densitometry (DEXA) within two years prior to study entry
No change in dose or route of hormones for six months
Normal mammogram within three months of RPFNA.
Biomarker Qualifiers for Trial Participation
RPFNA = Epithelial hyperplasia +/- atypia with Ki-67 ≥1.5%
RNA Extraction plus storage for later qRTPCR; History, breast and physical exam

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Study Entry Visit (within three months of RPFNA)

CBC chem. profile, fasting lipid profile, C-reactive protein,
bioavailable testosterone and estradiol,
serum bone turnover markers (osteocalcin and N-telopeptide),
25-OH Vitamin D levels; Mammogram digitized for density measurement
Complete hot flash scores, brief fatigue inventory, BCPT Symptom Checklist and HAQ Fibromylagia Questionnaire; History, breast and physical exam (if not previously done)
Randomization to letrozole 2.5 mg/day or placebo;
while continuing same dose of hormone replacement

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monthly phone contacts

↓

Six Month Study Visit

CBC chem. panel, fasting lipid profile, CRP, bioavaiable estradiol and testosterone,
osteocalcin and N-telopeptide, 25-OH Vitamin D levels, reserve serum for letrozole levels
Mammogram, density measurement
Repeat questionnaires, history and physical,
RPFNA and associated biomarkers; DEXA scan if not continuing on open label

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Optional Open Label Letrozole 2.5 mg/d

↓

monthly phone contacts

↓

12 Month Study Visit (for women choosing open label letrozole)

Repeat assessments performed at six month visit; DEXA scan at completion

Goal: 96 evaluable subjects at six month visit

1.0 Objectives

Our primary hypothesis is that proliferation and expression of other estrogen response genes in hyperplastic breast tissue obtained by RPFNA from high risk women on chronic HRT will be favorably modulated by six months of letrozole relative to placebo without substantially increasing hot flashes or worsening overall quality of life.

To test our hypothesis, we have formulated the following objectives:

1.1 To determine whether Ki-67 in hyperplastic foci of breast epithelium obtained by random periareolar fine needle aspiration from postmenopausal high-risk women on HRT is reduced by six months of letrozole.

1.2 To determine if letrozole causes an increase in hot flashes, fatigue, pain, stiffness; a deterioration in overall quality of life; as well as deleterious changes in bone turnover markers, total cholesterol:HDL ratio, and C-reactive protein.

1.3 To determine if morphologic characteristics as assessed by nuclear morphometric measurements and semiquantitative Masood index score improve with six months of letrozole.

1.4 To determine if six months of letrozole in women on a stable dose of hormone replacement results in consistent down modulation of estrogen responsive genes, particularly pS2 and GREB-1.

At the conclusion of this proof of principle study, we will have established a firm clinical and biological basis for a large randomized study of letrozole versus placebo in high-risk postmenopausal women requiring hormone replacement for symptom control with development of breast cancer as an endpoint. If aromatase inhibitor treatment is found to reduce risk of breast cancer in women receiving hormone replacement, it would provide middle age women with tools to alleviate perimenopausal symptoms while minimizing breast cancer risk.

2.0 Background

2.1 Need For Development of Prevention Intervention for Postmenopausal women on HRT for Control of Menopausal Symptoms

It is estimated that one quarter of postmenopausal women in the United States take hormone replacement therapy [National Institutes of Health 2005]. This is primarily because nothing else is as efficient in relieving perimenopausal symptoms [Barton 2002; Nelson 2004]. There is no identified primary prevention strategy for those women who are both at high risk for breast cancer because of family history or prior pre-cancerous biopsy and who need hormone replacement therapy to alleviate perimenopausal symptoms [Fabian 2005C; Wickerham 2006]. Standard drug prevention therapy for postmenopausal women is tamoixfen or raloxifene and major prevention Phase II clinical trials in postmenopausal women are investigating use of aromatase inhibitors [Cuzick 2005]. Current expert opinion and ASCO guidelines suggest that hormone replacement not be used with tamoxifen [Vogel 2001; Chlebowski 2002] and clinical trials with aromatase inhibitors (AIs) or raloxifene exlude women who use hormone replacement therapy. Concerns about side effects and quality of life are major reasons why the majority of women have avoided primary prevention therapy with anti-estrogenic drugs in the past [Port 2001; Vogel 2002; Tchou 2004; Bober 2004]. Advice to a symptomatic high risk woman that she discontinue hormones in order to take anti-estrogens has a poor likelihood of being heeded as this would force her to both give up an effective therapy for her menopausal symptoms in addition to starting a medication which has a substantial chance of worsening them. An effective prevention strategy which would permit symptomatic high risk women to continue taking hormone replacement is needed and is the subject of this protocol.

2.2 Rationale for Use of Aromatase Inhibitors in High Risk Post-Menopausal Women Taking Hormone Replacement Therapy

The rationale for use of aromatase inhibitors as primary prevention therapy in high risk women taking hormone replacement comes from six lines of evidence.

1. Up-regulation of aromatase in areas of benign breast disease which is common in high risk women.

2. Breast aromatase acitivity is responsible for the majority of breast tissue estrogen in postmenopausal.

3. Aromatase inhibitors have been shown to be more effective than tamoxifen in adjuvant trials in preventing contra-lateral cancers in postmenopausal women.

4. Pre-clinical data in aromatase over-expressing animals suggests the aromatase inhibitor, letrozole, is effective in a high estrogen environment.

5. Low dose estrodiol may prevent some side effects associated with aromatase inhibitors.

6. Our pilot study of 6 months of letrozole in high risk postmenopausal women on a stable dose of hormones showed a substantial reduction in proliferation in hyperplastic epithelium obtained by random periareolar fine needle aspiration without a significant chane in serum estradiol or increase in perimenopausal symptoms.

These lines of evidence are explored in more detail below.

2.2.1 Up-regulation of Aromatase in Benign Breast Disease as Well As Breast Cancer

Aromatase which converts androgens to extrogens is present not only in the ovary but in breast, muscle, brain, bone, uterus, blood vessels, and other organs especially adipose tissue. Aromatase enzyme promoter sensitivity differs by organ type. In the ovary the aromatase enzyme promoter is exquisitely sensitive to increases in follicle stimulating hormone (FSH). The aromatase promoter in the breast is minimally sensitive to FSH but very sensitive to inflammatory cytokines and growth factors such as IL6, TGFβ and TNFα and prostaglandin E2, most of which increase with age (e.g. IL6), or proliferative breast disease, and cancer [Santen 1999, Miller 2001; Yue 2001, Brueggemeier 2003]. Thus aromatase activity is often up-regulated in proliferative breast disease as well as breast cancer [Brueggemeier 2003].

2.2.2 Aromatase Activity Responsible for Much of Breast Tissue Estrogen in Postmenopausal Women

The majority of breast estrogen is derived from local production not systemic uptake [Santen 1999]. The large amount of aromatase in breast tissue may be one of the reasons that breast tissue levels of estradiol are 10-50X that of serum levels in postmenopausal women who are not on HRT [Santen 1999]. Aromatase inhibitors have traditionally not been used for treatment of systemic metastases in premenopausal women as in this setting they produce only a partial blockade of estradiol synthesis by the ovary. The reflex rise in LH and FSH, plus the large amount of the aromatase substrate androstenidione, allows the premenopausal ovary to continue estradiol synthesis at about 60% of baseline despite aromatase inhibitor treatment [Santen 1980]. Nonetheless, there have been anecdotal reports of responses in premenopausal women with metastatic breast cancer to the first generation AI, aminoglutethimide [Wander 1986; Santen 1989]. Clinical response to AIs in postmenopausal women with established breast cancer has been directly correlated with breast tumor aromatase activity, and this association seems to be stronger than for estrogen receptor levels [Miller 2001].

2.2.3 Aromatase Inhibitors More Effective Than Tamoxifen in Reducing Contra-Lateral Tumors in Adjuvant Trials

Aromatase inhibitors are associated with superior disease free survival and reduced contralateral new primary cancers compared to tamoxifen in adjuvant trials. Adjuvant trials comparing an AI (anastrozole or letrozole) with tamoxifen as initial endocrine treatment or switching to an AI (exemestane) after 2-3 years of tamoxifen are associated with an approximate 50% reduction in contralateral breast cancer in women with hormone receptor positive tumors [Coombes 2004; Thurlimann 2005; Howell 2005]. Neither tamoxifen or raloxifene is effective in reducing ER negative cancer incidence [Fisher 1998; Wickerham 2006], and neither reduces estradiol levels.

Estradiol activation of ERα increases production of several growth factors which may result in paracrine stimulation of growth of ER negative cells [Clarke 1997; Russo 1999; Shoker 1999]. Clinical evidence supporting estrogen depletion as a means of reducing the incidence of ER negative as well as ER positive tumors comes from studies of BrCa1 deleterious mutation carriers in whom oophorectomy substantially reduces the risk of breast cancer despite the fact that most BrCa1 mutation carriers develop ER- tumors [Rebbeck 1999, Eisen 2000; Rebbeck 2002]. Thus, AIs, by reducing breast estrogen synthesis, have the potential to reduce the incidence of ER negative as well as ER positive tumors [Santen 1999].

2.2.4 Low Dose Hormone Replacement May Prevent Some Side Effects Associated with Aromatase Inhibitors

In adjuvant trials women randomized to aromatase inhibitors had fewer thromboembolic events, uterine cancers and vaginal bleeding compared to tamoxifen [Coombes 2004; Thurlimann 2005; Howell 2005]. Women randomized to an AI had a similar incidence of hot flashes as tamoxifen and a higher incidence of arthralgias, bone mineral density loss, vaginal dryness, pain with intercourse, and loss of libido [Coombes 2004; Thurlimann 2005; Fallowfield 2004; Howell 2005].

2.2.4.1 Side effects of letrozole compared to placebo

Compared with placebo, extended adjuvant therapy with the AI letrozole was associated with a higher incidence of hot flashes, arthritis, arthralgias and myalgias. The incidence of osteoporosis was borderline higher for letrozole ($p=0.07$). Quality of life studies (SF-36 and MENQOL) indicated worsening of sexual function, bodily pain, vitality, and vasomotor symptoms for women randomized to letrozole versus placebo. Most of these symptoms can be explained by estrogen deprivation at the target tissue level [Fallowfield 2004].

2.2.4.2 AIs and Cardiovascular Disease

In adjuvant trials, there was an early non-significant trend toward a higher number of cardiovascular related events and deaths in women randomized to AIs compared to tamoxifen [Coombes 2004; Thurlimann 2005; Howell 2005], but generally, no increase in cardiovascular events when an aromatase inhibitor was compared to placebo [Goss 2003]. Tamoxifen's partial estrogen agonist effects on the liver result in favorable modulation of total cholesterol:HDL ratio [Love 1990], an important predictor of cardiac events [Ridker 2005]. In primary prevention trials the improved total/HDL cholesterol ratio observed with tamoxifen has not translated into a significant overall protective effect on the cardiovascular system [Fisher 1998], possibly because this is partially offset by an increase in triglycerides and a number of pro-coagulant molecules [Hozumi 1998; Brun 1986 Decensi 1998; Marttunen 2000]. Letrozole like other AIs has minimal effect on lipids when compared to placebo and when blood samples are obtained from fasting subjects [Harper-Wynne 2002; Goss 2003; Atalay 2004]. In a mature (5 year) analysis of the Intergroup Exemestane Study (IES), in which women undergoing adjuvant hormonal therapy were randomized to exemestane or further tamoxifen after 2-3 years of tamoxifen, women randomized to exemestane had an overall survival advantage without a significant increase in ischemic heart disease or stroke [Coombes 2006].

Although the results of the IES trial are reassuring, it is possible that AI-induced severe estrogen depletion might have adverse effects on vascular endothelial function which might explain the non-significant trend in ischemic cardiovascular events compared with tamoxifen [Thurlimann 2005; Everson 1995; Elisaf 2001; Blumel 2003]. In preclinical models, activated ERα in blood vessels facilitates smooth muscle relaxation and vasodilatation in response to nitrous oxide [Bolego 2005]. This response is abolished by estrogen deprivation [Bolego 2005]. If AIs reduce available tissue estradiol in blood vessels the result could be accelerated atherosclerosis [Nakamura 2005]. It is theoretically possible that the use of low dose estradiol with an AI might prevent or blunt the effects of estrogen deprivation on vascular endothelium similar to the protective effects on bone turnover markers.

2.2.4.3 AIs and Osteoporosis

While it is still unclear whether use of AIs increase risk of clinical heart disease, it is very clear they increase the risk of osteoporosis, bone fracture, arthralgias, and joint stiffness [Cuzick 2005]. Increase in the risk of osteoporosis may in part be due to the unfavorable effect of estrogen depletion on vitamin D and joint physiology. Development of osteoporosis in women taking AIs may be due to unmasking of subclinical vitamin D insufficiency with estrogen depletion due to down regulation of the 1,25-α hydroxylase enzyme which converts circulating inactive provitamin D to the active form, and down regulation of the vitamin D receptor

[Reichel 1989; Heaney 2005]. The result is reduced absorption of calcium and bone mineral loss due to elevation of parathormone.

Kendall et al. from the Royal Marsden Hospital studied 10 women given letrozole for 6 weeks then letrozole plus a quarter of an estradiol patch for weeks 6-16. Serum estradiol levels increased to 12 pmol with the patch. Serum crosslaps, a marker of bone turnover and predictor of bone mineral density loss, which had increased the first 6 weeks on estradiol alone returned towards normal with add back estradiol [Kendall 2006]. Arthralgias and morning stiffness are observed after menopause with increased frequency and have been presumed to be due to estrogen deprivation. Articular cartilage contains estrogen receptor α and β and physiologic changes have been observed in primate studies in proteoglycan synthesis and in IGFBP-2 following estrogen administration [Richmond 2000].

2.2.4.4 Risks and Benefits of Hormone Replacement Therapy

Hormone replacement therapy, while associated with a reduction in menopausal symptoms, osteoporosis and hip fracture as well as colon cancer, is also associated with increased risk of breast cancer and venous and arterial thromboembolism [Rossouw 2002; Anderson 2004; Hays 2003; Peeyananjarassri 2005]. Risk varies with type, dose and route of drug administration as well as age of the individual. For breast cancer, the increase in relative risk is 1-2% per year of use for estrogen alone, and 5-10% per year for estrogens plus progestins [Chlebowski 2003; Anderson 2004; Beral 2003; Fournier 2005]. No increase in risk of breast cancer has been demonstrated for women who began oral premarin alone in their 50's and continued for an average of eight years [Chlebowski 2003]. Increase in risk of breast cancer applies to current users only and may be less for micronized progesterone than a synthetic progestin in women taking combination therapy [Fournier 2005].

Increase in risk of cardiovascular events and deep venous thrombosis appear to be related to route of administration, type of preparation as well as a woman's age. Increase in deep venous thrombosis and cardiovascular events and dementia is probably due to the procoagulant effects of oral estrogen and progestins promoted by increased production of thrombophilic proteins as a result of a first pass effect of estrogen through the liver [Anderson 2004].

Younger women ages 50-60 on estrogen alone may actually experience a reduction in coronary disease risk due to the favorable properties of estrogen on vascular endothelium [Anderson 2004; Hsia 2006; Blumel 2003]. There appear to be fewer procoagulant effects [Modena 2005; Langer 2005] and thromboembolic events [Oger 2003; Scarabin 2003] with transdermal compared to oral preparations. C-reactive protein along with lipids are powerful predictive biomarkers for future cardiovascular events among healthy women [Ridker 1998, 2005]. Specifically the increase in C-

reactive protein and other intermediate markers of inflammation observed with oral preparations are not observed after transdermal estradiol [Modena 2005; Langer 2005; Fournier 2005; Rossouw 2002; Manson 2003; Lowe 2000, 2001; Post 2003]. Although there is increasing use of the more physiologic transdermal preparations [NIH 2005], it still accounts for a small minority of prescriptions and consequently it is difficult for us to mandate that women eligible for this study be on this type of hormone replacement. However, if letrozole were found to reduce breast cancer risk in women on hormone replacement and the thromboembolic complications could be reduced by use of transdermal formulations, the potential impact on quality of life for a large number of menopausal high risk women could be substantial.

Further, for those high risk women contemplating primary risk reduction with an AI the combination of the AI plus low dose hormones might be as effective as antihormonal primary prevention therapy, with fewer side effects than with AIs alone.

2.3 Preclinical Evidence Supporting Use of Letrozole in a High Estrogen Environment

Aromatase over-producing transgenic mice spontaneously develop mammary hyperplasia and dysplasia which may progress to cancer if a carcinogen is also administered [Tekmal 1999]. Letrozole reduces ER expression, proliferative indices, and reverses dysplasia in hormonally intact animals [Kirma 2001; Luthra 2003]. Thus, in a preclinical model, letrozole appears effective in reversing precancerous changes in a moderately high estrogen environment.

2.4 Biomarker Based KUMC Phase II Pilot Study Suggests Reduction in Proliferation in Benign Breast Disease in Postmenopausal Women Given Letrozole Despite Continuing HRT

2.4.1 Use of Risk Biomarkers as Response Endpoints in Phase II Chemoprevention Trials

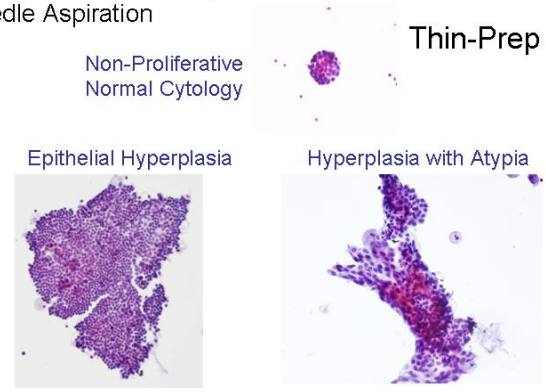
Several potentially reversible risk biomarkers are used as indicators of response in phase II prevention trials. These include intra-epithelial neoplasia, and related molecular markers such as proliferation, mammographic breast density, serum bioavailable estradiol and testosterone, and serum insulin growth factor one (IGF-1) and its binding protein IGFBP3 [reviewed in Fabian 2005A]. Many of these biomarkers are interrelated such that pre-menopausal women with high breast density may be more likely to have higher serum levels of IGF-1 and IGFBP3, and a greater chance of underlying proliferative breast disease [Byrne 2000; Boyd 1995, 1992, 2002].

Not all of these biomarkers are good risk or response indicators for postmenopausal women. For post-menopausal women, serum IGF-1 and IGFBP3 do not appear to be good surrogates of breast tissue levels of these factors and are not associated with risk as in pre-

menopausal women [Hankinson 1998]. Likewise breast density may not be a good response biomarker in postmenopausal women either because they have little density at baseline and/or little reduction has been observed with standard prevention therapy such as tamoxifen [Cuzick 2004]. For example, there was only a 1% median reduction of breast density observed with tamoxifen in women in the IBIS-1 trial over the age of 55 compared with marked reduction in density in women under age 45. Further, there may actually be an inverse relationship between some postmenopausal risk factors such as body mass and mammographic density [Boyd 2002]. Thus it is not clear that breast density is a useful response biomarker for postmenopausal women [Cuzick 2004].

Precancerous changes are common in high-risk women undergoing prophylactic mastectomy [Hoogerbrugge 2003], but the majority of high-risk women have never had a diagnostic biopsy. We have previously demonstrated that random periareolar fine needle aspiration (RPFNA) detected hyperplasia with atypia (Figure 1) increases the risk for DCIS or invasive breast cancer by 5 fold in high risk women and significantly stratifies risk assessments based on the Gail model alone [Fabian 2000; Mayo 2001]. Although atypical epithelial cells obtained by diagnostic biopsy, nipple aspirate fluid cytology, or random periareolar fine needle aspiration are associated with an approximate 5 fold increase in risk relative to those without atypia [Hartmann 2005; Wrensch 2001; Fabian 2000], no improvement in benign breast disease morphology has been shown after 6-12 months of treatment with tamoxifen or other SERMS [Mohsin 2005, Fabian 2006].

Figure 1: Cytomorphologic Characterization of Specimens Acquired by Random Periareolar Fine Needle Aspiration



2.5 Use of Proliferation as a Primary Response Biomarker in Breast Cancer Treatment and Chemoprevention Trials

Proliferation as measured by detection of mitotic index and/or detection by immunohistochemistry of nuclear associated proliferation antigens such as Ki-67 (MIB-l) progressively increases from normal to hyperplasia to atypical hyperplasia and DCIS [Shrestha 1992, Mommers 1999]. In

normal, non-proliferative breast tissue, Ki-67 is expressed in approximately 0.2%-0.7% of cells in the terminal lobular duct unit but has been reported in 1-6% of cells in proliferative breast disease [Shaaban 2002, Pavelic 1992, Allred 1998]. Shaaban et al. in a correlational cross-sectional study have reported that high Ki-67 in foci of hyperplasia (median 3.8%) is associated with a greater risk of breast cancer than those with low Ki-67 (median 0.8%) [Shaaban 2002]. We have observed a median Ki-67 labeling index of 1% in RPFNA samples from high-risk postmenopausal women exhibiting hyperplasia without atypia and a median Ki-67 of 2.8% in RPFNA samples containing hyperplasia with atypia [Khan 2005]. A number of antihormonal treatments effective in breast cancer treatment and/or prevention have been shown to reduce proliferation indices in benign and cancerous breast epithelial cells by 17-50% relative to baseline [Clarke 1993, Fernando 1994, Chang 2000, Dowsett 2000, Dowsett 2001, Dowsett 2005A, Decensi 2003]. Estrogen withdrawal has been directly correlated with a 5-fold reduction in Ki-67 in MCF-7 xenografts [Detre 1999]. Reduction in proliferation (Ki-67) at two weeks and 12 weeks in the neoadjuvant IMPACT Trial (tamoxifen, anastrozole, combination) was positively associated with disease free survival [Dowsett 2005A]. Tamoxifen has been shown to reduce proliferation (Ki-67) in benign breast tissue of postmenopausal women [de Lima 2003]. For predictive value, Ki-67 must be reduced by at least 35-50% by the intervention [Urruticoechea 2005].

At the present time, change in proliferation as measured by Ki-67 is probably the most commonly used primary response indicator used in moderately sized proof-of-principal Phase I and II breast chemoprevention trials in high risk postmenopausal women [Fabian 2005A].

2.6 Design of Pilot Study of Letrozole

We have completed a single arm pilot study of six months of letrozole in high-risk women who had been on a stable dose of hormone replacement therapy for at least six months prior to baseline random fine needle aspiration and continued their hormones throughout the study period. Change in proliferation as assessed by Ki-67 immunohistochemistry was the primary study endpoint.

Eligibility

Subjects were required to be at high risk as defined by one or more of the following criteria: 1) five year Gail risk >1.67%; 2) known BRCA1/2 mutation carrier; 3) prior contralateral treated DCIS; 4) prior biopsy showing LCIS or atypical hyperplasia. Postmenopausal status was defined as having either a prior bilateral oophorectomy, prior hysterectomy with ovaries intact and age greater than 50, or prior natural menopause with no period for at least 12 months prior to baseline RPFNA. Potential subjects must have been in good health with an ECOG performance status of 0-1, reasonably normal organ function, a baseline serum estradiol of <150 pg/ml, and a normal mammogram performed within six months prior to the baseline RPFNA.

The subject's baseline random RPFNA must have exhibited hyperplasia with atypia or borderline atypia with a Masood quantitative index score of ≥14.

In addition, there must have been ≥ 500 ductal cells on the cytomorphology slide, and ≥500 ductal cells on the slide designated for Ki-67 staining with evidence of Ki-67 expression. They must also have a third slide with at least 100 ductal cells evaluable for ER staining.

2.7 Methods for Pilot Study

RPFNA and Cytomorphology

RPFNA was performed under local anesthesia from two sites from both breasts and all cells pooled from both breasts (one breast in the case of prior DCIS or invasive cancer) into a single 15 cc tube containing modified Cytolyt™ (9 cc Cytolyt™ and 1 cc 10% neutral buffered formalin). At least three slides were prepared by a standard ThinPrep 2000 (Cytyc) non-gynecologic protocol. Slides for cytomorphology, Ki-67, and ER were first Pap-stained under RNase free conditions. Cytomorphology assessment was performed by a single cytopathologist and classified by three different methods traditional [Zalles 1995], Masood semiquantitative index score [Masood 1990], and the 1996 National Cancer Institute Consensus Panel Criteria [The Uniform Approach 1997]. Masood index scores between 6-10 are generally associated with non-proliferative specimens, 12-13 hyperplasia and 15-18 with hyperplasia with atypia. Scores of 11 are generally borderline between non-proliferative and hyperplasia and scores of 14 borderline between hyperplasia and atypia. We have previously found that use of the Masood scoring system reduces intra-observer interpretive variance [Fabian 2002]. Consequently, the Masood score index was used to determine change in cytomorphology. A change of 3 or more points is generally consistent with a descriptive category change and thus considered significant.

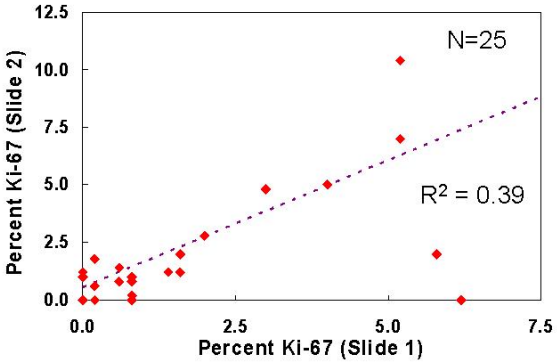
Ki-67 Assessment

Only baseline slides containing >500 epithelial cells were processed and stained for Ki-67. Hyperplastic clusters were preferentially assessed and the number of cells with unequivocal nuclear staining out of 500 cells assessed was recorded. Slides were hand scored by two readers and assessments recorded on case report forms. In case of a disagreement between two readers, assessments were averaged for the final score; however, agreement for Ki-67 scores between the two readers is generally excellent with Cronbach's α=0.99 [Khan 2005].

Reliability and Reproducibility of Ki-67

Change in Ki-67 between duplicate slides and over 6 months in a high-risk group not undergoing an intervention is being assessed in an AVON-NCI funded Breast SPORE grant. We have information on 25 subjects for which 2 Ki-67 slides were made from different aliquots of the same specimen. There appears to be good reproducibility (Figure 2).

Figure 2: Correlation between Duplicate Slides for Assessment of Ki-67



Estrogen Receptor Assessment

Estrogen receptor expression was assessed with ID5 antibody from Dako diluted at 1:100 and applied for 30 minutes. The ER protocol was optimized by us for fine needle aspirate specimens processed as ThinPrep slides [Petroff 2006A]. This protocol involved use of low temperature (90°C) retrieval for two minutes with a 0.2X nuclear decloaker (Biocare) and use of a 0.01% glucose oxidase blocking reagent for 30 minutes at 37°C. ER was hand scored by two readers utilizing 100 cells from a hyperplastic duct exhibiting ER staining. The proportion of cells staining at each intensity (0-4+) was assessed. The proportion of cells staining at each intensity was then multiplied by the intensity to achieve proportion/intensity sub-scores. The weighted intensity score (IS) was computed by adding together the proportion/intensity sub-scores. Under this system scores can theoretically range from 0-4. However, for benign hyperplastic cells which average 30% ER expression with 2+ staining, our median intensity score was approximately 0.6.

Hormones and Growth Factors

In addition to cellular assessments, blood was obtained for estradiol, progesterone, testosterone, SHBG, and IGF-1/IGFBP-3 at baseline and at six months and frozen at -80°C until analysis. Samples were not obtained under fasting conditions. Estradiol and testosterone were measured in serum by EIA following ether extraction [England 1974; Pearce 1989]. Ether extraction is used to avoid false elevations of estradiol due to large circulating amounts of estrone sulfate. IGF-1 and IGFBP-3 were measured in serum by ELISA. Baseline and six months samples were assessed in the same run.

Mammographic Breast Density

Mammograms were digitized for breast density assessment by the computer-assisted Cumulus program developed by Boyd and Yaffee [Boyd 1995]. Baseline and six month films were assessed together by a single reviewer in a blinded fashion.

qRT-PCR

We have recently established the feasibility of quantitative real time reverse transcription PCR to allow the measurement of a panel of biomarker genes from Cytolyt-formalin fixed breast epithelial cells

processed per our normal RPFNA protocol and harvested by laser capture microdissection [Petroff 2006B]. Briefly, 50-500 breast epithelial cells are harvested from a recently Pap-stained slide using the Autopix platform and Paradise kits from Arcturus. mRNAs are selectively amplified through a single round of linear amplification with T7 polymerase, converted to cDNA, and then stored at -80°C until analysis by real time PCR using Taqman chemistry.

Adverse Events

Adverse events were collected using Common Toxicity Criteria. In addition, a hot flash score index was assessed at baseline using methodology previously published by the Mayo Clinic in which the number of hot flashes/day is multiplied by the usual intensity [Loprinzi 2002]. Subjects were contacted monthly for adverse event reporting.

Accrual

We planned to enter 45 subjects over a period of 24 months in order to obtain 40 subjects evaluable for change in Ki-67. With this accrual, we anticipated being able to detect an effect size for change in Ki-67 of 0.48 SD with 80% power at a type I error rate of 5%.

2.8 Results of the Letrozole Pilot Study

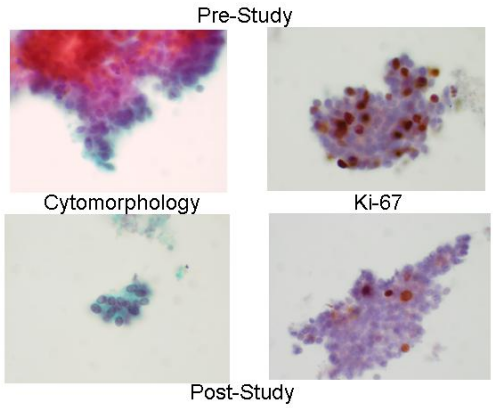
Forty-two subjects were accrued in 23 months. Accrual was stopped early when it was apparent that the early dropout rate was negligible and that we would have sufficient subjects with Ki-67 to meet our goal. Forty-two subjects have completed trial, with one subject discontinuing letrozole at 4 months but she did undergo repeat RPFNA and is therefore evaluable.

Table 2 summarizes results for the 42 subjects enrolled on the study. Median age at baseline was 50 (range 39 to 68), with 7% of subjects over 60. The median five year Gail risk was 2.2%. Thirty-five of 42 subjects were taking estrogen alone (various preparations) and seven estrogen plus a progestin. Fifty-nine percent were on transdermal preparations. The median duration of hormone replacement therapy was two years (range 0.5-21 years). Median baseline estradiol was 57 pg/ml and median Ki-67 4.0% (range 0.3 to 23.6%). Median ER weighted intensity score (IS) was 0.66; 36/42 subjects had >10% cells which exhibited ER expression in hyperplastic foci. Median baseline Masood score was 14 (range 14 to 16).

Table 1. Pilot Trial Results for 42 Women Enrolled on Study			
	Median Values		
Variable/Biomarker	Baseline	Post-Study	Change (relative)
Age, years	50		
5-Year Gail Risk, %	2.2		
Cytomorphology, Masood Score	14	14	-1
Ki-67, %	4.0	1.5	-2.3 (-0.66)
ER, %	40.5	50.0	0.0 (-0.07)
ER, IS	0.66	0.60	0.0 (-0.09)
Mammographic Density, % area	31.5	29.4	-0.4
Estradiol, pg/ml	57.4	47.6	-7.5
Testosterone, ng/ml	0.58	0.56	-0.07
Progesterone, ng/ml	3.22	2.6	-0.28
SHBG, nM	55.8	51.5	0.48
IGF-1, ng/ml	172	181	6.3
IGFBP-3, ng/ml	4281	4396	79
IGF-1:IGFBP-3 molar ratio	0.15	0.15	0.002

After six months of letrozole, a reduction in Ki-67 was observed in 31/42 (74%) subjects, while continuing with baseline hormone replacement. The median pre- and post- study Ki-67 was 4.0% and 1.5%, respectively (Table 1). The mean absolute change in Ki-67 was –2.3%, with 95% confidence intervals (-4.2% to -0.8%) which did not encompass zero. The median relative change was -66% of the baseline values. Reduction in Ki-67 was observed for 27 of the 35 subjects taking estrogen alone and for 4 of the 7 subjects taking estrogen plus a progestin. As an example of the robust response possible with 6 months of letrozole, Figure 3 shows cytomorphology and Ki-67 expression for the pre-study and post-study RPFNA specimens. For this subject, not only was cytology changed from hyperplasia with atypia to simple hyperplasia, but Ki-67 was reduced from 13.2% to almost 0.

Figure 3: Effect of 6 Months of Letrozole



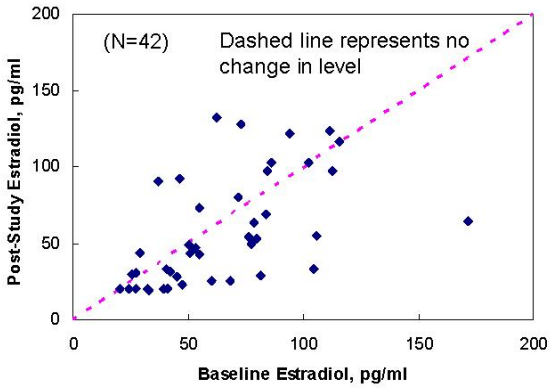
Our results differ from those published by Harper-Wynne et al. for a small pilot study of letrozole [Harper-Wynne 2002]. They found no significant decrease in Ki-67 after 12 weeks of letrozole in core biopsy specimens of benign breast tissue from high risk postmenopausal women. However, their median baseline Ki-67 was only 1.25%. Seven of our subjects had a Ki-67 <1.5%. Six of these seven exhibited no decrease after six months of letrozole. This underscores the inability to detect change at very low levels of Ki-67 as has been reported by others [Harper-Wynne 2002; Dowsett 2005A].

Six subjects had essentially no baseline staining for ER in areas of hyperplasia (ER IS score <0.10). Baseline Ki-67 ranged from 0.8 to

23.6%, and Masood scores from 14 to 16 in these subjects. All subjects without baseline ER expression, except one who also had a low baseline Ki-67 of 0.8%, exhibited a dramatic reduction in Ki-67 at six months. These findings in benign breast tissue are similar to those of Miller et al. [Miller 1987] and Yue et al. [Yue 2001] who found that aromatase expression is similar in ER- and ER+ breast cancer; and that aromatase activity is a stronger predictor of clinical response to AIs than is ER [Miller 1987, 2001].

There were no significant changes in cytomophology, serum estradiol (Figure 4), testosterone, progesterone, SHBG, ER intensity score, or mammographic density although small reductions were seen for Masood score, estradiol, testosterone, and progesterone (see Table 1). There was no change observed in IGF-1. Results for IGF-1 and ER are similar to those reported by Harper-Wynne et al. [Harper-Wynne 2002] for high risk postmenopausal women not taking hormones.

Figure 4: Estradiol Change with Six Months of Letrozole



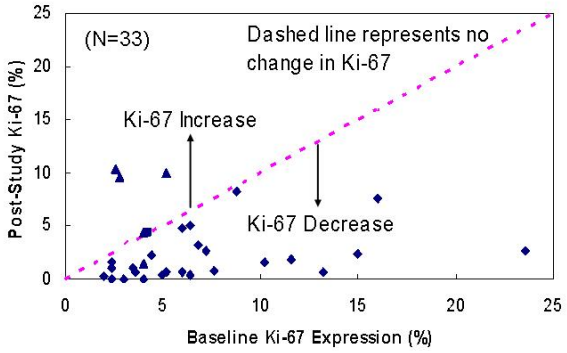
Reported side effects included hot flashes, arthralgias, fatigue and temporary hair thinning. Median change in hot flash score was 0 (–8 to +9). Seventeen percent reported an increase, 14% a decrease, and 69% reported no change in hot flashes over the duration of the study. Thirty-eight percent reported arthralgias and 41% fatigue when questioned on a monthly basis. The regimen was well-tolerated and only one subject discontinued study prematurely (after 4 months). This is in comparison to the ongoing National Cancer Institute of Canada MAP.2 trial of exemestane vs placebo, where 5 of our accrued 32 subjects discontinued study within the first few months primarily due to hot flashes and related symptoms.

We performed a sub-analysis utilizing the 33 subjects whose baseline Ki-67 was ≥2% and who were taking estrogen with or without a progestin, i.e., those who meet the eligibility criteria for the proposed Phase II trial. The baseline, post-study, and change values (Table 2) were similar to those of the entire group of 42 subjects.

Table 2. Pilot Trial Results for 33 Women Taking E or E+P, with Baseline Ki-67 ≥2.0%			
	Median Values		
Variable/Biomarker	Baseline	Post-Study	Change (relative)
Age, years	49		
5-Year Gail Risk, %	1.9		
Cytomorphology, Masood Score	14	14	-1
Ki-67, %	5.0	1.6	-3.0 (-0.83)
ER, %	41.0	50.0	-7.0 (-0.11)
ER, IS	0.70	0.53	-0.04 (-0.12)
Mammographic Density, % area	32.8	32.6	0.9
Estradiol, pg/ml	59.9	46.7	-7.4
Testosterone, ng/ml	0.60	0.57	-0.07
Progesterone, ng/ml	3.5	2.5	-0.3
SHBG, nM	57.1	51.5	0.3
IGF-1, ng/ml	185	187	6.6
IGFBP-3, ng/ml	4291	4409	81
IGF-1:IGFBP-3 molar ratio	0.16	0.16	0.005

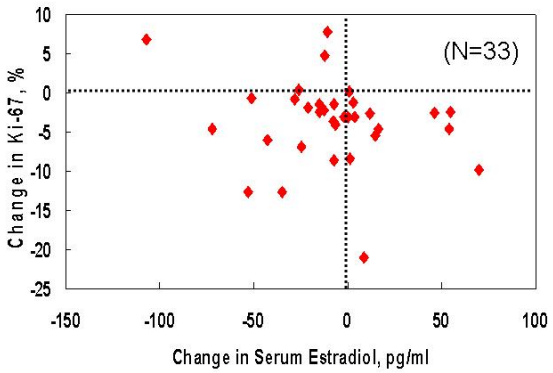
Median baseline Ki-67 was 5.0% and median six month Ki-67 was 1.6%. The median absolute change was a reduction of 3.0% (95% CI, -5.6% to -1.7%). The relative change was an 83% reduction (95% CI, -75% to -10%). A decrease in Ki-67 was exhibited by 28 of the 33 subjects (Figure 5). Median change in Masood cytomorphology index was –1 (range -5 to +1).

Figure 5: Ki-67 Change with Six Months of Letrozole



Most critically, there was no correlation between the change in serum levels of estradiol and changes observed for Ki-67 expression in RPFNA specimens (Figure 6).

Figure 6: No Correlation between Change in Estradiol and Change in Ki-67



qRT-PCR Analysis of Gene Expression

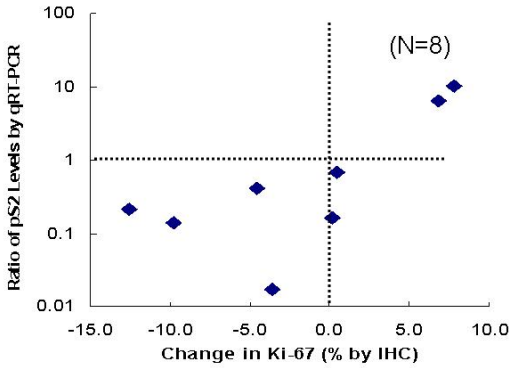
For the pilot study, primer probes with a 3' bias were constructed for ER, aromatase, pS2, PCNA, MCM2, Cyclin D1, survivin, and VEGF. The quantity of RNA is expressed relative to the level of that for a housekeeping gene. The housekeeping gene differed by type of marker to be detected. Markers expected to be in relatively high abundance (e.g., ER, Cyclin D1) were tested against actin which is highly expressed. Markers expected to be expressed in relatively low levels (e.g., proliferation markers, pS2, aromatase) were tested against TATA box binding protein (TBP), a housekeeping gene that is expressed at low levels. PCR reactions were run on an Applied Biosystems Prism 7000 Sequence Detection System. PCR values obtained with crossing threshold (CT) values if fewer than 30 cycles were considered valid for analysis [Giulietti 2001; Feldman 2002; Gjerdrum 2004; Goldworthy 1999; Liss 2002; Al-Taher 2000; Liviak 2001; DeCremoux 2003].

We began harvesting RNA for qRT-PCR midway through the trial using an IRB approved ancillary protocol. Only 21 subjects had baseline and 6-month material harvested on cells remaining after 3 slides had been prepared, as per the main protocol. Initially, there were several technical challenges in modifying the standard Arcturus protocol to maximize good quality RNA from formalin/Cytolyt fixed RPFNA samples. Microdissection and isolation of RNA should be performed within a few days of H&E staining. Further, with ThinPrep liquid processing, most of the desirable larger clumps of cells float to the edge of slide viewing area where it is not possible to employ laser-assisted microdissection. Nonetheless, in this exploratory substudy, we were able to identify 14 subjects with good quality RNA as confirmed by QC Metric following a 1:10 dilution of RNA. Eight genes plus two housekeeping genes were assessed on this material.

In general, ER was detected in all 14 subjects at both baseline and 6 months in fairly abundant amounts, aromatase at very low levels, and pS2 in low levels. The most informative marker appeared to be pS2 where CTs

were below 30 both at baseline and 6 months for 8/14 cases, and these 8 cases correlated well with change in Ki-67. Figure 7 plots the relative expression for pS2 (6 month value/baseline) vs. change in Ki-67 expression (6 months – baseline). It is encouraging that pS2 (an estrogen responsive gene) was generally reduced (values less than 1) when Ki-67 was decreased (negative values), supporting the hypothesis that we are lowering estrogen at the tissue level despite continuing systemic administration of hormone replacement therapy.

Figure 7: Comparison of Changes in Ki-67 vs. pS2 Expression.



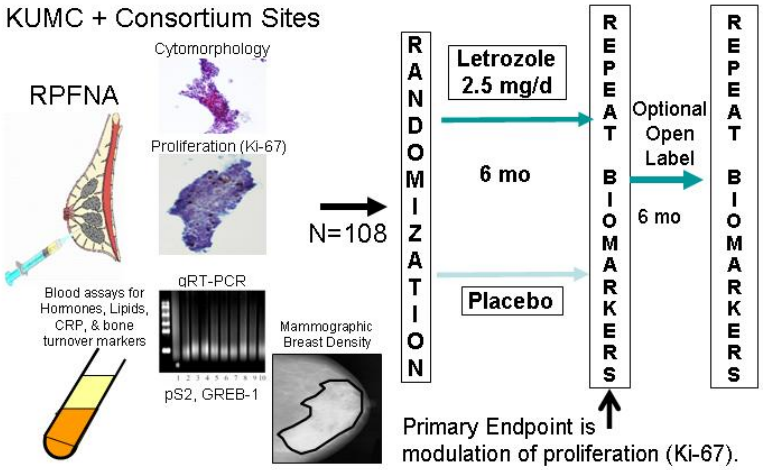
Nuclear Morphometry

A developmental study in collaboration with the University of Arizona was conducted to examine quantitative nuclear morphometry (karyometry) as a means of describing the proportion of abnormal cells before and after prevention intervention. Papanicolaou-stained ThinPrep slides of RPFNA samples from 11 subjects were digitally recorded at high resolution with 200 cells measured in each slide, at baseline and at 6 months. The nuclear chromatin pattern was assessed for its characteristics by multivariate analytic techniques; a determination of nuclear abnormalities was carried out and procedures were applied that allowed the identification of a subpopulation on cells which showed expression of abnormality. This provided an estimate of 6.6 % of abnormal cells at base line, and of 1.6 % at 6 months, indicating a decrease in the frequency of abnormal cells following treatment with letrozole. The slides from the remaining 31 subjects are being analyzed for a validation of this preliminary result. These results will provide the algorithm for blinded (sequence of slides and study agent assignment) analysis of specimens from the proposed trial.

2.9 Summary

Based on these encouraging pilot study results, we propose a multi-institutional, randomized placebo-controlled proof-of-principal trial (Figure 8) in postmenopausal high risk women taking hormone replacement therapy, with reduction in Ki-67 as the primary study endpoint.

Figure 8: Phase II Chemoprevention Trial of Letrozole for Post-menopausal Women on HRT at High Risk for Breast Cancer



3.0 Summary of Study Plan

This is a multi-institution double-blind placebo-controlled trial to whose main endpoint is to determine if 6 months of letrozole (2.5 mg daily) can reduce proliferation as assessed by Ki-67 in high risk postmenopausal women on systemic hormone replacement therapy who have RPFNA evidence of hyperplasia with atypia or borderline atypia, and a minimum Ki-67 in a hyperplastic foci of $\geq$ 1.5%. Secondary efficacy endpoints include assessment of change in morphology by nuclear morphometry and the semi-quantitative Masood score index and change in qRT-PCR assessed estrogen response genes (GREB-1, pS2). Mammographic density and bioavialable estradiol and testosterone will be assessed as well. No difference in change between letrozole versus placebo is expected for mammographic breast density, Masood Index score, or serum hormones, but significant reductions are expected in Ki-67, expression of estrogen response genes by qRTPCR, and the proportion of cells deemed abnormal by nuclear morphometry. Other secondary endpoints include change in biomarkers associated with bone and cardiovascular health, toxicity measurements using the NCI CTCAE version 3.0, BCPT Symptom Check List (Mayo Clinic) hot flash score, general fatigue inventory the Fibromyalgia Impact Questionnaire from the Health Assiessment Questionnaire, (which assessed joint stiffness and functional impact). These are validated questionnaires and it should take no more than 10-15 minutes to complete all three questionnaires. We predict there will be little difference in most parameters between placebo and letrozole at 6 months in these women continuing their HRT. Exceptions may be arthralgais, fatigue in a minority of women, particularly those with sub-optimal 25-OH vitamin D levels. We will be assessing baseline and six month 25-OH vitamin D levels to determine if women with an increase in bone turnover markers, fatigue and arthralgias are likely to have underlying vitamin D insufficiency which is unmasked by use of letrozole. Subsequent to the 6-month RPFNA for assessment of biomarkers, toxicity and quality of life assessments, all women may receive otional open-label letrozole for an additional 6 months, followed by a third RPFNA and biomarker assessment. The primary purpose of the subsequent 6 months open label letrozole is to facilitate recruitment, but also to assess biomarker and toxicity endpoint changes with longer treatment. A DEXA scan will be performed at completion of study to document any effects on bone health.

Number of subjects: The primary endpoint will be the change in Ki-67 expression at 6 months. Specifically, we will compare this change using a two-sample t-test between subjects randomized to letrozole versus those randomized to placebo. From our pilot study, the average percentage of cells expressing Ki-67 at baseline was 6.4% with an average change of -3.7% with a common standard deviation of 5.5% in subjects meeting the eligibility criteria for this trial. Using a 1 to 1 randomization scheme and presuming mean change for placebo is 0, we will have 90% power to detect a difference of 3.7% in Ki-67 in the letrozole group with a type I error rate of 5% with a total of 96 evaluable subjects. We intend to accrue 108 subjects in order to attain 96 evaluable subjects.

The clinical flow design we will use to satisfy the Specific Aims is as follows:

←Screening period→ -3 to 0 months	←——— Treatment period ———→ 0-12 months					
VISIT 1 High Risk Postmenopausal Subjects on ERT for > 6 months with stable dose and route DEXA within 2 years Breast Exam, Mammogram (for breast density) RPFNA (cytology, Ki-67) Hyperplasia +/- atypia and Ki-67 >1.5% CBC, chem, 25-OH-D Baseline history/ Breast exam, Physical*	VISIT 2 Day 0 of drug/ placebo dosing	Monthly phone contacts	VISIT 3 Month 6	Monthly phone contacts	VISIT 4 Month 12	OFF STUDY PHONE CONTACT
	Enter study Baseline history/ Breast exam, Physical* bioavailable estradiol, testosterone 25-OH-D Fasting lipid profile Osteocalcin N-teleopeptide Surveys Questionnaires	Trial coordinator contact subject via telephone evaluate AE's, compliance	Repeat baseline studies, return unused study agent or placebo, evaluate AE's, compliance, dispense open label drug DEXA scan if completing study	Trial coordinator contact subject via telephone evaluate AE's, compliance	Repeat baseline studies, return unused study agent, evaluate AE's, compliance DEXA scan	Trial coordinator contact subject via telephone evaluate AE's

***Baseline history, breast exam and physical may be done at any point within 3 months prior to randomization and starting study agent. If done prior to Visit 2, any new conditions or changes will be evaluated and documented before randomization and starting study agent.**

4.0 PARTICIPANT SELECTION

4.1 Inclusion Criteria for Screening RPFNA

Note that the screening RPFNA is conducted under a separate IRB-aproved research protocol with its own consent form. Potential subjects are not required to make a decision regarding participation in the letrozole trial until after receipt of the results of their RPFNA.

4.1.1 Menopause Status

4.1.1.1 Postmenopausal women at increased risk for breast cancer, age 30-69, on a stable dose of systemic HRT for six or more months.

Postmenopausal status defined as having either a prior bilateral oophoerctomy, prior hysterectomy with ovaries intact and age greater than 50, or prior natural menopause with no period for at least 12 month prior to baseline RPFNA

4.1.2 Hormone Replacement

On a well-absorbed systemic estrogen or estrogen and progestin preparation at least six months (women receiving only poorly absorbed vaginal estrogen such as vagifem or estring, or those receiving a testosterone plus estriol vaginal gel do not qualify). Dose and type of hormone replacement must be stable for at least six months.

4.1.3 Minimum Risk Criteria

- Gail 5-year Risk $\geq$1.7% and/or relative risk of $\geq$3X that for the average woman of the same age group, as calculated by the NCI Breast Cancer Risk Assessment Tool (http://www.cancer.gov/bcrisktool/Default.aspx); or a 10-year relative risk of $\geq$ 3X that for the population, as calculated by the IBIS Breast Cancer Risk Evaluation Tool (http://www.ems-trials.org/riskevaluator/);
- Known to carry BRCA1/2 mutation characterized as deleterious or of uncertain significance;or family history consistent with hereditary breast cancer
- Prior ADH/DCIS/LCIS; or hyperplasia with atypia on RPFNA.

4.1.4

Subjects must have had a baseline mammogram performed within three months prior to screening baseline RPFNA, interpreted as not suspicious for breast cancer, with any supplementary imaging required performed and negative prior to RPFNA.

4.1.5

Subjects must have had bone densitometry (DEXA) within two years of entry onto the study that demonstrated a normal bone mineral density (BMD). T-score must be greater than -1.0; or -1.5 to -1.0 if on active osteoporosis treatment.

4.2 Exclusion Criteria for Screening RPFNA

- Started or stopped or changed dose of hormone replacement within 6 months;
- Receipt of any aromatase inhibitor or selective estrogen receptor modulator (tamoxifen, raloxifene, arzoxifene, etc.) within the prior 12 months.
- Participation on any chemoprevention trial within the past 6 months
- Bilateral breast implants
- Current anticoagulant use
- Any other condition or intercurrent illness that in the opinion of the investigator makes the subject a poor candidate for RPFNA
- Invasive breast cancer or other invasive diagnosis within five years
- Metastatic malignancy of any kind
- NSAIDS use within prior three weeks

4.3 Inclusion Criteria for Study Entry

- RPFNA hyperplasia +/- atypia (Masood score of ≥13), Ki-67 ≥1.5% positivity (≥500 cells), extra cells for qRTPCR studies (estimated by lab technician from remaining amount in Preservcyt Container).
- Willing to continue the type, dose and route of their hormonal regimen at baseline aspiration throughout the duration of the study participation (6 months for randomization portion, 12 months if participates in the optional open label period);
- Have reasonable normal organ function before being dispensed study agent/placebo, as defined as:

Platelets	>100,000/dL
Hb	>10 g/dl
Absolute granulocyte count	>1000/ul
Creatinine	<1.5 mg/dl
Albumin	>3.0 g/dl
Bilirubin	<2.0 mg/dl
AST	<2 times upper limit of normal
Alkaline Phosphatase	<2 times upper limit of normal

- Have serum level of 25-OH vitamin D of at least 30 ng/ml prior to study entry.
- Willing to have a repeat RPFNA and mammogram at 6 months and 12 months (if participates in the open label portion of the study) following initiation of study drug.
- Willing to undergo a history, breast and physical exam prior to randomization/starting study agent and at 6 months and be contacted monthly by the trial coordinator during the 6 month study period (12 months if participates in the open label portion of the study);
- Willing to have approximately six tubes of blood drawn at baseline and six months (12 months if participates in open label portion of the study);
- Willing to sign and able to understand separate consents for the RPFNA's and study participation;

4.4 Exclusion Criteria for Study Entry

- Prior history of osteoperosis or osteoperotic fracture
- Prior History of Invasive Breast Cancer or other invasive cancer within five years from date of study entry
- Current and chronic use of Cox-2 Specific Inhibitors or NSAIDs, defined as seven consecutive days (1 week) for greater than three weeks during the six months prior to RPFNA
- Receiving treatment for rheumatoid arthritis or fibromyalgia
- Current history of poorly controlled migraines or perimenopausal symptoms-
- Currently receiving other investigational agents.
- Receipt of more than 6 months of an aromatase inhibitor (anastrozole, exemestane, letrozole, etc.) at any time in the past.

4.5 Inclusion of Women and Minorities

All women who meet eligibility criteria and members of all races and ethnic groups are eligible for this trial.

4.6 Recruitment and Retention Plan

Potential subjects will be screened in the Breast Cancer Prevention Center at the University of Kansas Medical Center (KUMC), Kansas City, Kansas. Or in similar clinics at the other participating institutions. Accrual duration is expected to take 42 months, accruing 2.5 subjects/month. It is expected that for every 4 postmenopausal subjects taking hormone replacement therapy screened by RPFNA, 1 of those will be eligible for participation.

Subjects will be contacted by phone during the months between clinic visits in order to insure active participation and to collect adverse event, dosing and other pertinent information. It is expected that the dropout rate will be less than 5%.

5.0 AGENT ADMINISTRATION

Intervention will be administered on an outpatient basis. Reported adverse events and potential risks are described in Section 9.2.

5.1 Dose Regimen and Dose Groups

Letrozole 2.5 mg or letrozole placebo will be given to all trial participants for the first 7 months and open label letrozole the second 6 months. All subjects will be given eight months (one month of overage) of drug at the baseline and 6 months visit to allow for scheduling study procedures. All subjects should have off-study procedures no later than within 36 hours of the last dose of letrozole or placebo. However, if a participant stops study drug prematurely in a unscheduled situation, she should come in for biomarker re-assessment as early as possible.

5.2 Study Agent Administration

The Institutional Investigational Pharmacy will be responsible for receiving, inventory and dispensing of study agent.

A study number will be assigned to each participant and provided to the investigational pharmacy. Study agent will be dispensed with the study number of each subject. Seven months of study agent will be dispensed. The study coordinator will then give the study agent to the subject with instructions to take one capsule at approximately the same time of day in conjunction with intake of food and at least eight ounces of water and to keep track of missed doses or lost capsules.

5.3 Concomitant Medications

Subjects are required to maintain any hormonal use throughout the duration of study participation. Subjects are strongly encouraged to continue any routine medications throughout the duration of the study to avoid confounding adverse events due to starting and stopping other medications while on study. Subjects are not allowed to start tamoxifen, raloxifene, anastrozole, letrozole or any other SERM or aromatase inhibitor (or other chemopreventive) while participating on this trial.

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

5.4 Dose Modification

Dose modifications will not be allowed on this study. Because study agent is only available in a single dose capsule, no dose modification option is available. If a subject is experiencing adverse events or is having a medical procedure that requires a brief ***suspension*** of letrozole, this must be noted in the CRF on the dose suspension page. At the discretion of the

PI, subjects may be removed from participation for significant dose suspension time periods.

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

Subjects who experience grade 3-4 toxicity will have the study agent suspended. If the investigator is certain that the toxicity is not due to the agent, the agent may be re-instituted after resolution of the adverse event, providing:

1) the subject will have had a minimum of three months of study agent prior to biomarker assessment at six or twelve months;

2) the investigator feels relatively certain taking letrozole or participating in the study will not affect recovery from the condition

5.5 Interruption or Discontinuation of Treatment

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

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

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

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

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

5.6 Emergency procedure for unblinding

Any adverse event or situation that requires immediate unblinding is subject to the mutual consideration of the Protocol Chair and the Trial Statistician.

5.7 Adherence/Compliance

Each subject will be given a seven month supply of drug/placebo at trial entry and instructed to return all excess drug/placebo to investigator at the six month visit. Six months of dosing is the expected amount of dosing, however, seven months of drug/placebo will be given to each subject to accommodate scheduling issues. At the end of treatment the actual quantity of unused drug/placebo will be compared to the anticipated amount of unused drug. At the six month visit, all subjects have the option to receive seven months of open label drug supply, expected to cover six months of dosing and allow some flexability with scheduling the repeat RPFNA while on study drug.

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

5.8 Drug Records

Drug records regarding receipt, distribution and disposition of study drug will be maintained by the institutional investigational pharmacist. The packing slip will indicate from and to whom study drug/placebo was shipped, date, quantity, and batch or lot number. The dispensing record will indicate quantity and dates that study drug was dispensed to and return by each subject. At the completion of the study, all unused study drug/placebo will either be returned to Novartis or destroyed per Novartis instructions. The record documenting study drug return will include date, quantity, batch or code number, and the name of person/department to whom the drug was returned.

6.0 LETROZOLE INFORMATION

6.1 Letrozole IND Sponsor

Letrozole is an approved agent for treatment of breast cancer and will shortly begin evaluation in The NSABP4 trial as an agent for the primary prevention of breast cancer in postmenopausal women not on hormone replacement therapy. Since in this instance letrozole is being used in an experimental manner, we will file for an IND exemption, as we did for the pilot study.

6.2 Mechanism of Action

Letrozole is a non-steroidal aromatase inhibitor (4, 4'-1H-1,2,4-Triazol-1 ymethylene dibenzonitrile) with a molecular weight of 285.31. It is supplied by Novartis in 2.5 mg tablets for oral administration.

6.3 Pharmacokinetics

Letrozole is rapidly absorbed from the GI tract, and absorption is not affected by food. Letrozole is slowly metabolized to an inactive metabolite whose glucouride metabolite is renally excreted. About 90% of letrozole is recovered in urine. Plasma concentrations at steady state are 1.5 - 2 times higher than after a single dose, but after steady state is reached there is no accumulation over time. Letrozole is weakly protein bound and has a large volume of distribution. No change in letrozole pharmacokinetic parameters have been noted with age or moderate renal impairment. Subjects with moderate hepatic dysfunction had 37% higher mean AUC levels than those without impaired function.

6.4 Pharmacodynamics

Letrozole inhibits aromatase by competitively binding to the heme moiety of cytochrome p450 resulting in a reduction of estradiol and estrone synthesis in all tissues.

In premenopausal animals, letrozole decreases ovarian steroidogenesis, reduces uterine weight, increases LH but not FSH, results in regression of estrogen dependent tumors but does not alter adrenal mineral corticoid or glucocorticoid synthesis.
In postmenopausal women letrozole suppresses conversion of testosterone to estradiol and androstenedione to estrone in the fat, adrenal glands and other tissues. Treatment of postmenopausal women reduces serum estrone, estradiol and estrone sulfate but does not alter adrenal corticosteroid, aldosterone, or thyroid hormone synthesis.

6.5 Drug/Drug Interactions

No drug/drug interactions have been noted with warfarin or cimetidine. Concomitant use of tamoxifen and letrozole may decrease letrozole levels.

6.6. Clinical Indications

Letrozole is currently approved as first and second line therapy of metastatic breast cancer, and for use in the adjuvant setting instead of tamoxifen or after 5 years of tamoxifen in a dose of 2.5 mg per day. In the adjuvant setting, letrozole is given for 5 years

6.7 Adverse Reactions

6.7.1 Compared to placebo in adjuvant setting

Adverse events which occurred more significantly more frequently in the MA.17 trial of extended adjuvant letrozole versus placebo following 5 years of tamoxifen were: hot flashes (net differerce + 4%), loss of appetite (net difference + 2%), myalgias (net difference + 3%), arthralgias (net difference + 4%), alopecia (net difference +2%) [Goss 2003].

6.7.2 Compared to tamoxifen in adjuvant setting

In adjuvant trials women randomized to aromatase inhibitors had fewer events, uterine cancers and thromboembolic phenomena compared to tamoxifen [Coombes 2004; Thurlimann 2005; Howell 2005]. However, women randomized to an AI had a similar incidence of hotflashes and a higher incidence of arthralgias, bone mineral density loss, vaginal dryness, pain with intercourse, and loss of libido compared to those taking tamoxifen [Coombes 2004; Thurlimann 2005; Fallowfield 2004; Howell 2005].

6.7.3 Compared to tamoxifen in metastatic setting

In a study of 455 women with metastatic breast cancer with a median exposure time of 11 months the incidence of adverse experiences was similar for Femara (letrozole) (n=455) and tamoxifen (n=455). The most frequent adverse events were nausea (15%), hot flushes (18%), fatigue (11%), dyspnea (14%) and arthralgias (14%). Constipation was noted in 9%, headache 8%, diarrhea in 7%, vomiting in 7%, decreased appetite 4%, weight decrease in 6%, alopecia 5%, lower limb edema in 5%, and hypertension 5% (package insert). Discontinuation of drug for adverse experiences was performed for 2% of patients taking Femara (letrozole) and 3% of those taking tamoxifen. A similar distribution of adverse events has been reported for subjects randomized to letrozole vs placebo in the MA17 trial

6.8 Availability

Both letrozole and matched placebo are to be provided by Novartis to the investigational pharmacy at each participating Institution.

Both letrozole and letrozole placebo supplied in bottles of 100 tablets.

6.9 Agent Distribution

Agent will only be released after documentation of IRB approval of the KUMC-approved protocol and consent.

200 tablets will be dispensed at entry of the subject onto the study so as to provide an adequate number of capsules for 7 months. The 7 months is based on the planned 6 months of administration, plus approximately one additional month if required for scheduling of procedures or travel.

6.10 Agent Accountability

The Site Principal Investigator, or a responsible party designated by the Site Principal Investigator, must maintain a careful record of the inventory and disposition of all agents using a Drug Accountability Record Form (DARF). The Site Principal Investigator is required to maintain adequate records of receipt, dispensing and final disposition of study agent. This responsibility has been delegated to the Investigational Pharmacy at each participating site. Include on receipt record from whom the agent was received and to whom study agent was shipped, date, quantity and batch or lot number. On dispensing record, note quantities and dates study agent was dispensed to and returned by each participant.

6.11 Packaging and Labels

Letrozole and letrozole placebo will be supplied by Novartis in a form ready for dispensing. Each bottle will be labeled with a one-part label identifying study specific information, such as Study title, HSC protocol number, dosing instructions, recommended storage conditions, the name and address of the distributor, randomization number, and a caution statement indicating that the agent is limited by United States law to investigational use only and the agent should be kept out of reach of children.

6.12 Storage

Storage should be in a locked cabinet with limited access, at room temperature.

6.13 Registration/Randomization/Consent

Potential subjects will be registered for the screening portion of the study by site study coordinator and principal investigator on a site screening log. Potential subjects will be given a unique screening number by the site staff and this will be affixed to all eligibility specimens and documents that will be sent to KUMC for review. No PHI should be attached to these items.

Site staff will not randomize a subject until verification by KUMC Trial Coordinator by means of a faxed request (standard form provided for this purpose which will include an eligibility checklist and notification of acceptable consent) for a particular potential subject. The designated KUMC Trial Coordinator will fax back to the Site Coordinator an approval to randomize the subject based on the review of the eligibility checklist provided by the site.

Approved eligibile subjects will be randomized by a randomization schedule at each site, prepared and provided by the Study Statistician. The site staff and subjects will be blinded as to the assignment to drug or matched placebo.

The subject will be assigned a randomization number, after stratification by site and type of hormone replacement therapy. The randomization schedule will be stratified by site (each site will be provided a unique list for use only at that site), and whether the subject is on an estrogen preparation alone, or estrogen plus a progestin preparation. Randomization will be in sequential order according to the randomization list provided. Subjects approved for randomization will be assigned a site specific randomization number, consisting of four digits. The first digit will identify the site (to be determined later) and three digits identifying the specific potential subject.

Sub-contracted sites other than KUMC may use the study consent for the screening RPFNA, or use a research/clinical consent for that purposes. Subjects are not officially on study until they have made it through the entire eligibility process. KUMC will use the High Risk Protocol Consent (KUMC HSC#4601) for the purposes of the screening RPFNA.

6.14 Blinding and Unblinding Methods

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

6.15 Agent Destruction/Disposal

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

7.0 CLINICAL EVALUATIONS AND PROCEEDURES

7.1 Schedule of Events

Study Procedure	Eligibility Period	Treatment Period					
	Visit 1 Baseline	Visit 2 On-Study	Monthly Phone Contacts	Visit 3 6 month Visit	Monthly Phone Contacts	Visit 4 12 month visit (optional participation)	4 week follow-up phone contact
Eligibility Checklist	X						
Informed Consent*	X	X		X		X	
Medical History	X°	X°					
Breast Exam	X°	X°		X		X	
Physical Exam	X°	X°		X		X	
Mammogram	X			X		X	
Ht/Wt/Vital Signs	X	X		X		X	
RPFNA	X			X		X	
CBC, Chemistry, 25-OH vitamin D +	X	X		X		X	
#Fasting blood for: Bioavailable estradiol, testosterone, SHBG, osteocalcin, N-telopeptide, CRP, lipid panel	X			X		X	
#Trough blood (serum) sample				X		X	
Hot flash assessment	X	X	X	X	X	X	X
Fatigue inventory, BCPT Symptom Check List, HAQ Fibromylagia Impact Questionnaire		X		X		X	
Adverse Events		X	X	X	X	X	X
Dispense Study Agent/ Evaluate Compliance		X		X		X	
Concomitant Medication	X	X	X	X	X	X	X
Return Study Agent				X		X	
Off-Study DEXA scan				X	or	X	
Off-Study evaluation							X

*Subjects will sign a consent each time an RPFNA procedure is performed (at KUMC HSC#4601). Only subjects qualifying for the drug/placebo dispensation will sign the study consent document. For sites other than KUMC, they may determine to use the study consent for the screening RPFNA per the site IRB requirements. Subjects will not be officially "on study" until eligibility is determined.

#After a 10-12 hour fast, four gel-clot 7 ml tubes drawn, allowed to clot at room temperature for 30 minutes, spun at 3500 rpm for 15 minutes, and aliquots of serum into 8 polypropylene storage tubes. Tubes to be labeled with subject initials, randomization number and date of draw. Tubes frozen at -80◦C and then shipped to KUMC on $\geq$ 5 lbs of dry ice, with pre/post specimens shipped together. Notify KUMC by fax and email prior to shipment.

+CBC, Chemistry, and 25-OH vitamin D to be performed by local laboratory at the expense of site PI (to be included in budget/subject costs)

° Study medical history, breast and physical exams may be done at any point within 3 months prior to randomization and starting study agent. If done prior to Visit 2, any new conditions or changes will be evaluated and documented before randomization and starting study agent.

7.2 Pre-Study Procedures and Evaluations

7.2.1 Pre-study Eligibility RPFNA Procedure Details

RPFNA will be performed, under local anesthesia from both breasts (two sites on each breast) and all cells pooled from both breasts (one breast in the case of prior DCIS or invasive cancer) into a single 15 cc tube containing modified Cytolyt™ (9 cc Cytolyt™ and 1 cc 10% neutral buffered formalin). After cleansing the breast with betadine and alcohol swabs, the breast is anesthetized with epinephrine and lidocaine using a tuberculin syringe. After the surface has been anesthetized, epinephrine and lidocaine are injected deeper into the breast at two sites, approximately at the 10 and 2 o'clock locations. Then, 5-6 needle passes with sterile RPMI prewetted 10-12 cc syringes/with 21 gauge 1 ½ inch needle, within the anesthetic puncture sites to withdraw epithelial cells lining the ductal tree. Clinician will preferentially sample areas in which resistance (density) is appreciated with the tip of the needle. Sample is immediately expelled into a 15 cc plastic tube containing 9 cc of Cytolyt™ (Cytyc Corp) and 1 cc of 10% neutral buffered formalin. Fluid from the Cytolyt™ tube should be drawn into the needle/syringe hub approximately 4x when expelling material into tube solution, and subject cell material should not be expressed against the side of the tube. Tubes are then placed on a tube rocker for approximately 12-24 hours prior to mailing to KUMC.

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

Subjects are offered Ativan 1 mg prior to the procedure for anxiety, and analgesics after the procedure in order to control any pain that may occur. These medications are optional. Subjects should also be instructed to eat a full breakfast the morning of the RPFNA, to abstain from vitamin E, NSAIDS and fish oil products for three weeks prior to RPFNA, and if planning on taking Ativan, to have someone with them to drive them to and from the clinic.

Subjects meeting the eligibility criteria from the RPFNA procedure and completing other pre-study procedures will be offered participation in the trial.

7.2.2 Other pre-study eligibility procedures (see Eligibility in section 4.0)

- Risk assessment

- Mammogram not suspicious for cancer, and any follow-up imaging recommended completed and not suspicious for cancer

7.3 Baseline Studies

7.3.1 Brief Physical and Medical History

Subjects will have a brief physical (including clinical breast exam) performed by a study-associated clinician. All abnormal findings will be recorded on the physical source document, including duration and severity. Vital signs, height, and weight. Percent body fat will be recorded for those institutions with appropriate scales in the clinic.

Subject will have a medical history collected by study personnel, including event dates, duration, and severity.

Physical (including clinical breast exam) and Medical History can be performed up to 3 months prior to randomization and starting study agent, but will be confirmed at on-study visit and evaluated.

7.3.2 Bloodwork

Standard CBC/Chemistry (and 25-OH vitamin D for determination of eligibility) will be collected and analyzed in a local CLIA approved lab at the expense of the site investigator. All materials will need to be obtained by individual study site. CBC/Chemistry/25-OH vitamin D may be done at any time up to six weeks prior to day of randomization. Results must be known prior to subject randomization.

Fasting blood (10-12 hour fast) will be collected in four gel-clot serum tubes for a lipid panel, bioavailable estradiol and testosterone, bone turnover markers (osteocalcin and N-telopeptide crosslinks), high sensitivity C-reactive protein, 25-OH Vitamin D and processed, stored and shipped per section 7.0 and 8.0. Assays will be performed centrally by KUMC. Kits and explicit instructions will be provided for this bloodwork by KUMC to individual sites.

An additional 3 cc specimen will be collected for possible letrozole blood levels. See section 7.0 and 8.0 for processing, storage and shipping instructions. Assays will be performed centrally after shipment to KUMC. Kits and explicit instructions will be provided for this bloodwork by KUMC to individual sites.

7.3.3 Baseline symptom assessment

A baseline symptom assessment will be made via the BCPT symptom checklist, including such relevant conditions as hot flashes, menstrual abnormalities, pelvic pain, muscle and joint pain, and fatigue.

We will utilize the hot flash scoring system developed by Dr. Charles Loprinzi at Mayo Clinic. For muscle and joint symptoms and function we will use the validated HAQ Fibromyalgia Impact Questionnaire from the Health Assessment Questionnaire (Stanford), and for fatigue the brief fatigue inventory [Wolfe 2004; Mendoza 1999; Loprinzi 2002]. Adverse events will be recorded using NCI common terminology criteria for adverse events version CTCAE 3.0.

7.4 Evaluations During Study Intervention

Monthly Telephone Assessment

Subjects will be contacted by the study coordinator on a monthly basis to review dosing and adverse events. Subjects who are difficult to reach or do not respnd to messages and other means of contact may be considered non-compliant. These will be recorded in the source documents and subsequently entered onto CRFs.

7.5 Evaluation at Completion of Randomized Portion of Study at 6 Months (See section 7.1 for Study Calendar)

If necessary for synchronizing scheduling of RPFNA with continued use of letrozole, study agent may be continued for up to one additional month, until the RPFNA is performed. If this occurs, then studies associated with the repeat RPFNA and completion of study will be conducted at that time, not at a calendar six months.

Subjects should NOT take the study drug the day of the procedure and should have blood drawn after a 10-12 hour fast.

Subjects will have a mammogram, RPFNA, CBC/Chem and fasting study bloodwork collected, and a brief physical (including clinical breast exam) performed by a study-associated clinician (see the study calendar, section 7.1 for a comprehensive list). All abnormal findings will be recorded on the physical source document, including duration and severity. Vital signs, height, and weight will be recorded. Percent body fat will also be and percent body fat will also be recorded.

Bloodwork for CBC/Chem (sent to local CLIA lab), fasting (10-12 hour fast) bloodwork and drug trough serum sample will be collected.

Subjects wishing to participate an additional six months in the open label portion of the study will be given an additional 7 month supply and then asked to return for a repeat RPFNA, breast density and other biomarker assays (see study calendar section 7.1).

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

When the subject has completed taking study agent, she will receive a DEXA scan to document any effects on bone health.

7.6 Post-intervention Follow-up Period

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

8.0 BIOLOGIC SPECIMEN HANDLING, STORAGE AND SHIPPING

All specimens will be placed in polypropylene storage tubes labeled with subject initials and study/randomization number, date of collection and contents of tube.

RPFNA specimens are to be collected as in 7.1.1. Tubes should be placed on a rocker for 12-24 hours to ensure thorough bathing of cells. Tubes containing RPFNA specimens should be overnight shipped with cool packs and using the KUMC Outside RPFNA Shipping Form within 36 hours of collection. The shipping form should also be faxed to 1-913-588-3821 in order to notify the KUMC Lab of the shipment. A call to 913-588-3917 should also be made to make certain the lab is aware of the shipment.

CBC/Chem specimens and assays of 25,OH-vitamin D for eligibility will be sent to the local CLIA approved lab and the costs covered by the local PI budget.

Study bloodwork should be collected fasting in four gel-clot tubes, allowed to clot at room temperature for 30 minutes, spun for 15 minutes at 3500 rpm, and the serum poured into four polypropylene storage tubes and labeled as in 8.1. These specimens should be immediately frozen and stored at -80◦C until shipment to KUMC. All specimens for each subject should be sent together, overnight, on 5 lbs or more of dry ice, in order to be run together. Notification of KUMC via fax (913-588-3821) and phone (913-588-3917) must be made to make certain the lab is aware of the shipment.

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

9.0 SAFETY ASSESSMENTS

9.1 Definition of an Adverse Event

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

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

9.2 Adverse Event Reporting

9.2.1 Reportable Adverse Events

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

9.2.2 Adverse Event data elements

- AE onset date
- AE Verbatim Term
- CTCAE Term (version 3.0)
- AE end date
- Severity grade
- Attribution to study agent (relatedness)
- Reported as a Serious Adverse Event (SAE)?
- Action taken with study agent
- Outcome of the event
- Comments

9.2.3 Severity of Adverse Events

Identify the adverse event using the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. The CTCAE provides descriptive terminology and a grading scale for each adverse event listed. A copy of the CTCAE can be found at http://ctep.cancer.gov.

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

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

9.3 Assessment of Relationship of AE to Treatment

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

9.4 Follow-up of AEs

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

9.5 Serious Adverse Events

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

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

9.6 Reporting Serious Adverse Events

9.6.1 Central Reporting

KUMC will report all SAEs to Novartis, Pharmaceuticals Clinical Safety and Epidemiology Department (CS&E). NCI and to the FDA per GCP guidelines. These reports are to be filed utilizing the Form FDA 3500A (MedWatch Form). Documentation of this submission should also be forwarded to Novartis CS&E in a timely manner. The investigator must also notify Novartis CS&E department which of the SAEs has been expedited (15-day submissions) to the FDA, within 24 hours of submission to the FDA.

Novartis CS&E department must be copied on any study related safety information sent to the IRB.

In addition, any serious adverse event regardless of relationship to the study drug, including pregnancy, occurring in a subject after providing informed consent, while receiving study drug, and until four weeks after stopping study drug, must be reported to Novartis Pharmaceuticals CS&E Department by FAX (888-299-4565) within 24 hours of learning of it's occurrence. It is requested that SAEs are reported to Novartis using the Novartis PIND Serious Adverse Event Report form, with clear documentation in the narrative that the PI is the holder of a private IND. The period after discontinuing study drug may be extended if there is a strong suspicion that the drug has not yet been eliminated. All serious adverse events must also be reported for the period in which the study protocol interferes with the standard medical treatment given to a subject (e.g., treatment withdrawal during washout period, change in treatment to a fixed dose of concomitant medication).

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

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

9.6.2 Include the following information when contacting the Protocol Chair:

- Date and time of the SAE
- date and time of the SAE report
- name of reporter
- call back information
- affiliation/institution conducting the study
- protocol number

- title of protocol
- description of SAE, including attribution to drug and expectedness
- subject randomization number

9.6.3 Follow-up of SAE

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

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

10.0 BIOMARKER AND LABORATORY ASSESSMENTS

The RPFNA specimen will be analyzed in the KUMC Breast Cancer Prevention Center Laboratory, under the direction of Brian Petroff, DVM, PhD. The Breast Cancer Prevention Laboratory and Research Administrative Unit are located in the Lied Research Building. It consists of three laboratory rooms with a total of 1597 square feet plus 1200 square feet of office space. The laboratories are fully equipped for cell culture, the processing and assessment of cytology and immunocytochemistry slides, assay of serum hormones and IGF-1/IGFBP-3, (ELISA's RIAs), laser microdissection, qRT-PCR, and various other molecular biology techniques. Staff located in the administrative/laboratory unit include two cytology/immunochemistry technicians, and an additional technician experienced in performing ELISAs RIA's for hormone assays as well as RTPCR and other molecular assays.

10.1 Ki-67

Breast Cells from RPFNA are pooled in modified cytolyt and sent to KUMC by express mail within 72 hours of RPFNA and all will be processed to slides within 96 hours. Cells are processed to thin prep slides via standard manufacturer's specifications.

A minimum of three slides will be made by a liquid-based cytologic technique: at least one each for cytomorphology, Ki-67 and microdissected prior to RNA extraction. Total epithelial cells will be delineated with Mayer's hematoxylin counterstain and proliferating cells with a MIB-1 antibody for Ki-7. Only baseline slides containing > 500 epithelial cells are processed and stained for Ki-7. Antigen retrieval is performed with 10 nmol/L citrate buffer (pH 6) in a Biocare (Walnut Creek, CA) decloaking chamber for two minutes at 120 degrees C. Slides are then stained with a MIB-1 monoclonal antibody (M7240 Dako Cytomation, Carpinteria, CA) at a 1:20 dilution in a Dako Autostainer [Khan 2005]. Hyperplastic clusters are preferentially assessed and the number of cells with unequivocal nuclear staining out of 500 cells assessed and recorded. Manual counting will be employed and scoring assessed by two laboratory technicians. The scores will consist of number of cells assessed, and number of positive cells for Ki-67. A separate score for each technician and a consensus score will be recorded. The consensus score will be utilized for analysis of the primary endpoint. Representative cell clusters are archived with a digital imaging system.

In the event that fewer that 500 epithelial cells are present in the Ki-67 slide on the follow-up RPFNA, the cytology slide will be destained and used for Ki-67 staining. If there are still less than 500 evaluable cells present when all slide material is combined the post-treatment value for Ki-67 will be considered as the proportion of epithelial cells staining positive for Ki-67 relative to the total number of epithelial cells present. If no staining for Ki-67 is observed and there are less than 500 evaluable cells, the value will be imputed as the reciprocal of the total number of evaluable cells present. If there is no staining and greater than 500 cells, the value will be assumed to be zero. Subjects with less than 100 evaluable cells will be considered not evaluable for Ki-67.

10.2 Cytomorphologic Assessment

Cytomorphologic assessment will be performed as in the pilot study by Dr. Carola Zalles. She will provide both a traditional characterization (QNS, normal, apocrine metaplasia, epithelial hyperplasia, or hyperplasia with atypia) [Zalles et al 1995] and a semiquantitative cytology index score (modified Masood score) [Masood et al 1990] and a consensus panel (QNS, benign, intermediate/atypia, atypia/suspicious) description [The Uniform Approach to Breast Fine-Needle Aspiration Biopsy 1997]. The Masood semiquantitative index score will be the primary measure determining morphologic hange as we have observed that interpretive variance ($\geq$ 3 points) is less using this system [Fabian 2002].

10.3 Nuclear Morphometric Assessments

Nuclear Morphometric Assessments will be performed at the University of Arizona by Dr. Bartels and associates. Briefly, the cytomorphlogy slide is imaged, individual nuclei are acquired and stored in libraries. Morphometric analysis of approximately 40 features is performed to generate a cumulative score that reflects deviation from normality.

10.4 RNA Extraction and qRTPCR Assessments

RNA extraction will be performed after microdissection of the third slide using an Acturus Autopix and Macro LCM caps (Arcturus, Mountain View, California). RNA from microdissected epithelial cells and residual whole slide scraping is obtained using a commercial protocol (Paradise Reagent Solution, Arcturus) optimized for formalin-fixed specimens [Petroff 2006B]. Material is heated in an overnight incubation with proteinase K to lyse cells and dissociate nucleic acids prior to harvesting. RNA is isolated using column based chromatography and treated with DNase to eliminate any residual DNA. Purified RNA is amplified with a single round of T7 based linear amplification (Paradise Reagent System, Arcturus) and converted to cDNA and stored at -80°C until all specimens from a subject can be run in a single batch. Quality of RNA is checked by PCR (QC Metric, Arcturus). The quantity of RNA is expressed relative to the level of that for a housekeeping gene. The housekeeping gene differed by type of marker to be detected. Markers expected to be in relatively high abundance (e.g., ER, Cyclin D1) were tested against actin which is highly expressed. Markers expected to be expressed in relatively low levels (e.g., proliferation markers, pS2, aromatase) were tested against TATA box binding protein (TBP), a housekeeping gene that is expressed at low levels. PCR reactions were run on an Applied Biosystems Prism 7000 Sequence Detection System. PCR values obtained with crossing threshold (CT) values if fewer than 30 cycles were considered valid for analysis [Giulietti 2001; Feldman 2002; Gjerdrum 2004; Goldworthy 1999; Liss 2002; Al-Taher 2000; Liviak 2001; DeCremoux 2003].

The primary purpose of qRT-PCR assessments on estracted RNA is to a) confirm presence of the targets for letrozole (aromatase) and estradiol (ER); b) provide indirect evidence of reduction in breast tissue estradiol through assessment of the estrogen response genes pS2 (trefoil factor-1) and GREB-1 [Rae 2005].

10.5 Blood Assessments

10.5.1 Complete Blood Count and Metabolic Chemistry Profile will be performed at the participating sites at on-study, intermediate, and off-study visits. Results will be used to confirm absence of serious liver or renal dysfunction or blood dyscrasia.

Blood for all biomarker assays will be collected at the participating institutions in gel clot tubes, with serum separated according to standard procedures, 1-2 ml of serum aliquoted into six plastic tubes. These tubes will be labeled with the date and a study subject specific number, and then frozen and stored at -80°C until shipment on dry ice to KUMC. Assays on baseline, 6-month, and 12-month samples will be performed together in the same run to avoid batch variation.

10.5.2 Bioavailable Estradiol and Bioavailable Testosterone

We have extensive experience with use of serum hormones as biomarkers in clinical chemoprevention trials [Fabian 2002, 2004]. Serum levels of estradiol will be measured by sensitive radioimmunoassay at Ligand Assay and Core Analysis Laboratory at the University of Virginia following diethyl ether extraction at KUMC. The lower limit of detection at the Ligand Lab is 1.5 pg/ml and the intra-assay variation is 4.3%. Extraction prior to RIA avoids contamination by estrone sulfate and is often necessary to obtain accurate values in postmenopausal women on HRT [Dowsett 2005B]. Testosterone after diethyl ether extraction is also measured by a sensitive RIA with a lower limit of detection of 5 ng/dl and the intra-assay variation is 5%. SHBG will be assayed by an immunometric assay using the Immulite analyzer (Diagnostic Products Corp), with a lower limit of detection 0.2 nmol/L and intra-assay variation of 2.4%.

Bioavailable estradiol or testosterone is calculated from the fraction that is unbound to SHBG [Stumpf 1981]. Tracer amounts of tritiated estradiol or testosterone are added to aliquots of serum, ammonium sulfate is added to precipitate the SHBG with bound steroid, the sample is centrifuged, and then activity in the supernatant and the precipitate are measured in parallel by liquid scintillation counting. The percent activity in the supernatant is calculated to yield the fraction of bioavailable (non-SHBG bound) hormone. Sensitive chemiluminescent assays are being developed by the core analysis lab at the University of Virginia and may be used instead of RIA for analysis of estradiol and tesosterone [Dowsett 2005B].

We do not anticipate any significant difference in change in estradiol or testosterone between the letrozole and placebo groups.

10.5.3 Cardiovascular and Bone Fracture Risk Biomarkers

The combination of high sensitivity C-reactive protein and total cholesterol:HDL ratios provide excellent risk biomarkers for cardiovascular disease [Ridker 2005]. Lipid panel with total cholesterol:HDL ratio is performed on serum by an enzymatic assay after a 12 hour fast. High sensitivity C-reactive protein, an indicator of inflammation, will be performed by an immunochemiluminometric assay.

N-telopeptide crosslinks correlate with the rate of bone reabsorption and bone mineral density in osteoperotic women and can be used to monitor the efficacy of anti-reabsorptive therapy with estrogen and calcium [Greenspan 2000]. The test is performed on serum by EIA. Mean values for women are 12.6 nM BCE/L (6.2 – 19.0 nM BCE/L).

Osteocalcin is a measure of bone/reabsorption. It together with the N-telopeptide is helpful in determining reduction in bone turnover in response to anti-reabsorptive agents or predicting early bone mineral loss after hormone therapy is stopped. The test is performed on serum by an immunochemiluminometric (ICMA) assay [Slovik 1984]. It is best performed fasting as it cannot be performed on a lipemic specimen.

The above assays will be performed at LabCorp Inc. Research Division or a similar CLIA-approved laboratory.

Estrogen supplementation increases high density lipoproteins and thus it is possible that administration of letrozole will result in a higher total cholesterol:HDL ratio. We do not expect any difference in change in C-reactive protein, osteocalcin, or N-telopeptide between letrozole and placebo groups.

10.5.4 Serum 25-OH Vitamin D Levels

The cause of the increase and/or induction of joint stiffness and arthralgias in women taking aromatase inhibitors is not clear but may be due to estrogen deprivation in combination with suboptimal vitamin D levels. Blood levels of 25-OH vitamin D are a good measure of vitamin D adequacy and for optimum bone and general health should be in the 32-50 ng/ml range [Vieth 1999; Holick 2003; Heaney 2004]. Suboptimal or deficient levels of 25-OH vitamin D are common in postmenopausal women, ranging from 25 to 50% of those tested when 37 ng/ml is used as the lower cutoff [LeBoff 1999; Aguado 2000; Glowacki 2003]. Persistent pain, particularly in shoulders, wrists, hips, knees, legs, and ankles, as well as joint stiffness has been reported in 93% of individuals with 25-OH vitamin D levels <20 ng/ml [Plotnikoff 2003]. Taylor et al. has recently reported in abstract form that a high proportion of postmenopausal women on AIs with musculoskeletal complaints had vitamin D deficiency [Taylor 2004]. We will assess the effect of baseline and follow-up 25-OH vitamin D levels on subsequent development of arthralgias.

All samples will be run in in KUMC's clinical laboratory by a widely used RIA kit [Gunter 1988-1994]. The assay limit of detection is 1.5 ng/ml. Assay precision varies only slightly with vitamin D level from 8.6 to 12.5% for run-to-run variation and 8.2 to 11% for between-run variation.

We do not expect any increase or decrease in 25-OH vitamin D levels other than that expected from seasonal variations. However, low 25-OH vitamin D levels may explain development of arthralgias or an increase in bone turnover if encountered in women randomized to letrozole.

10.6 Breast Density Measurements

Mammograms will be performed within three months of the baseline aspiration and at six and twelve months. An attempt will be made to use the same machine at baseline and at 6 and 12 months. However, it is imperative that a woman has breast imaging performed on either analogue or digital for all three imaging procedures. Either copies of the mammograms or direct digital files will be sent to KUMC from collaborating sites for assessment of breast density. For assessment of mammographic breast density from analog films, the right and left cranial caudal views will be digitized using a Lumiscan 85 film digitizer.

These films will be used for a blinded assessment of breast density parameters using the Cumulus software program [Boyd 1998; Boyd 1997]. Both Dr. Fabian and Dr. Kimler have been trained by Dr. Boyd in Toronto in the use of this method. Specifically, Dr. Fabian achieved >85% agreement with Dr. Boyd on training.

Prior to the analaysis, images will be checked to make sure cropping (total breast volume) is similar. For the analysis, dates will be removed so the reader will not know which is the baseline and which is the 6 or 12 month film, but the three will be analyzed in the same batch. Stone et al have recently reported that the method of analysis with the least intraobserver variance is for the reviewer to view the initial and the follow-up mammograms together, but with the reviewer blinded as to which is the baseline and which is the follow-up study [Stone 2003] Both the absolute area of increased density and proportional density (area of increased density divided by total area of breast) will be assessed.

10.7 Quality of Life Evaluations

The most frequent adverse events in our pilot trial were arthralgias, fatigue and hot flashes. Consequently, in addition to utilizing the Breast Cancer Prevention Trial (BCPT) symptom scale (used as part of NSABP prevention trials) [Ganz 1995; Day 1999], we will use specific tools geared to the most prevalent symptoms in the pilot study.

The BCPT symptom checklist is a 42 item list of problems common to peri and postmenopausal women and those undergoing anti-hormonal

treatment in prevention trials. Women are asked if they have had the symptom in the past four weeks. If they answer yes, they are asked to grade the severity (0-4). This tool can be used to compute a total score or individual or related items of interest (e.g., three items relate to joint stiffness and arthralgias) may be scored [Stanton 2005].

The Mayo Clinic Hot Flash scoring system was developed by Loprinzi et al. Subjects are asked about their daily number of hot flashes and rate usual severity from 1 to 4. The frequency is multiplied by usual severity to obtain a total score [Loprinzi 2002].

We do not expect an increase in symptoms or hot flash scores for women randomized to letrozole.

The Fibromyalgia Impact Questionnaire (HAQ) is a validated tool routinely used by rheumatologists to evaluate pain, stiffness, and physical functioning. Individual domains or a total score can be utilized in assessment [Wolfe 2004].

The brief fatigue inventory asks the woman to rate her current as well as worst and usual fatigue in the past 24 hours. It also asks her to rate how the fatigue has affected mood, general activity, walking, work relations, and enjoyment of life [Mendoza 1999].

We do not expect an increase in joint pain, stiffness or fatigue for women randomized to letrozole, unless a woman has suboptimal levels of 25-OH vitamin D.

11.0 DATA MANAGEMENT

Participant data will be collected using protocol-specific case report forms (CRF) developed by KUMC. A copy of all documents up to the month six visit will be mailed to KUMC as soon as they are completed, and the remainder as soon as the subject completes study.

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

Source document files/chart must be kept available at sites for monitoring visits during the conduct of the study through data analysis.

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

Laboratory data from RPFNA and blood analysis perform at KUMC will be entered on source documentation, entered into a database and reviewed by accuracy by KUMC study staff.

Co-existent disease and adverse events will be coded using the NCI CTCAE, version 3.0.

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

11.1 Source Documents

Source documents will consist of reports (mammogram reports, laboratory reports, etc.), and forms created specifically for this study that will capture information such as physical exam, phone contact information, adverse events, concomitant medications, demographics, etc. To the extent which possible, source documents (aside from reports) will be formatted to be similar to the CRF format, in order to make the transcription from source to CRF more reliable. Special laboratory CRF's will be created to capture biomarker laboratory data including cytomorphology, nuclear morphometry mammographic density, Ki-67, serum hormone levels, osteocalcin, C-reactive protein, fasting lipid profile and 25-OH vitamin D levels. Data will be entered in an Excel spread sheet which can be merged with the database held at KUMC.

11.2 Data and Safety Monitoring Plan

Prior to activation of the clinical trial, a Data Safety and Monitoring Committee (DSMC) will be established. The DSMC will consist of four members that will be identified later: a clinical faculty member at KUMC, a member of the Clinical Trials Division of the Kansas Cancer Institute, and

two external members with experience in clinical prevention trials. One member will be selected by the Committee to serve as Chairman for administrative purposes.

The DSMC monitors data management, subject safety, and subject accrual. The DSMC also provides advice on the overall conduct of the study. Meetings will be held by phone on an annual basis. Interval conference calls may be conducted as required. The functions of the DSMC are primarily those of an overview nature in regard to both data and safety, as detailed below.

All data collected for this trial will ultimately reside in a central database. The sources of data are three-fold: (1) All clinical data that originate at KUMC or collaborating sites are entered onto specific Case Report Forms (CRFs). The CRFs will be audited and source documents reviewed by a central multi-site monitor, after which the CRFs will be sent to KUMC. The data are entered into the central database and audited/validated. Periodically, these data are output to individual subject summary sheets so as to allow periodic assessment by the Protocol Chair (Dr. Carol Fabian) and staff. (2) Biomarker data from the assessments at KUMC of fine needle aspiration specimens (cytologic characterization, cytology score, and Ki-67 and ER immunocytochemistry score) and mammographic breast density are entered onto trial specific CRFs. Data are then entered into a trial specific central database. There are appropriate checks (such as comparison of electronic files to validated paper printouts) on data accuracy and completeness for all sources of data. This is accomplished via inspection of summary reports prepared for the DSMC by the biostatistician for this project, Dr. Matt Mayo.

The subject cohort consists of essentially healthy women who are defined as being at high risk for the development of breast cancer, but do not have any clinical evidence of or suspicion for an existing malignancy. As such, side effects that might be due to the study intervention are expected to be minimal but should be easily identified and monitored.

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

In addition to the functions listed above, the DSMC may serve in a general advisory role in regards to assessing the progress that is being made on the study. Subject accrual rates, and specifically problems with accrual that might impact on the ability of the trial to be completed, can be addressed by the DSMC. Likewise, the committee may make suggestions on other aspects that would improve the quality of the trial.

11.3 Sponsor or FDA Monitoring

The NCI, DCP (or their designee), pharmaceutical collaborator (or their designee), or FDA may monitor/audit various aspects of the study. These monitors will be given access to facilities, databases, supplies and records to review and verify data pertinent to the study.

11.4 Record Retention

Clinical records for all participants, including CRFs, all source documentation (containing evidence to study eligibility, history and physical findings, laboratory data, results of consultations, etc.), as well as IRB records and other regulatory documentation will be retained by the Protocol Lead Investigator (Dr. Carol Fabian) in a secure storage facility in compliance with HIPAA, OHRP, FDA regulations and guidances, and NCI/DCP requirements unless the standard at the site is more stringent. The records for all studies performed under an IND will be maintained, at a minimum, for two (2) years after the approval of a New Drug Application (NDA). The records should be accessible for inspection and copying by authorized persons of the Food and Drug Administration. If the study is done outside of the United States, applicable regulatory requirements for the specific country participating in the study also apply.

12.0 STATISTICAL METHODS

The study is a Phase II, double-blind, randomized, placebo-controlled clinical trial to assess the potential efficacy of letrozole as a chemopreventive agent, as measured by changes in proliferation index (Ki-67) between baseline and 6-months. Secondarily, the study will evaluate other pharmacodynamic properties of the agent and assess which biomarkers, if any, are modulated. Side effects, adverse events, and quality of life will also be assessed.

Standard randomization strategies will be employed with type of HRT (E vs E+P) as the only stratification factor other than institutional site so that each site can be provided with a unique randomization schedule at initiation. All eligible subjects will be randomly assigned to letrozole or placebo in a 1:1 ratio.

12.1 Sample Size and Power

The primary outcome of interest is efficacy of letrozole relative to the placebo, thus the pre- to post-treatment change in a proliferation index (percent of cells expressing Ki-67) is the basis of the power analyses. From our pilot study, the average percentage of cells expressing Ki-67 at baseline was 6.4%, and the average change in percent of cells expressing Ki-67 was a 3.7% reduction, for subjects meeting the same eligibility requirements as will be used in this proposed trial.

A 1:1 randomization of 96 evaluable subjects will provide 90% power to detect an effect size of 0.67 standard deviations with a Type I error rate of 5% using a two-sample, two-sided t-test. In other words, if one assumes a common standard deviation of change of 5.5% (as observed in the pilot study for the 33 subjects matching eligibility criteria for the proposed trial) for both placebo and letrozole groups and if mean change for placebo is zero, then there will be 90% power to detect an average change of 3.7% in Ki-67 in the letrozole group with a Type 1 error rate of 5%. Note that the 3.7% reduction is what was seen in the pilot study. Using a conservative drop-out rate of 10% (it was 2% in the pilot study), enrollment of 108 subjects should provide the required 96 evaluable subjects for the primary endpoint.

12.2 Statistical Analysis

All analyses will be intent-to-treat. All subjects randomized will be evaluable for safety and all subjects that have both a pre and post specimen are evaluable for efficacy. Primary analysis of efficacy will be performed on all evaluable subjects and secondary analysis will be done on those subjects who received at least 3 months of study medication. From our experience we have very little missing data, however, if the missing data rate is above 5%, we will use multiple imputation to impute these values to perform the primary and secondary analyses.

The primary outcome will be change in Ki-67 (percent of cells expressing Ki-67) from baseline to 6 months. The two-sample t-test will be used to compare the change in Ki-67 between the letrozole and placebo groups. Any difference in baseline values of Ki-67 between the two groups would

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

For the secondary endpoints, similar methods as above will be used to compare the treatment and control groups at two time points for all markers, i.e., serum hormones, mammographic breast density, and clinical laboratory tests. We will also correlate change in the primary biomarker Ki-67 with change in other markers (e.g., bioavailable estradiol, pS2, GREB-1) using Pearson's correlation coefficient. Again, if there exists a high degree of skewness in these variables, Spearman's correlation will be utilized.

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

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

Evaluation of drug-induced hormonal changes - We will examine whether Letrozole has any effects on serum levels of bioavailable estradiol, and testosterone, determined at baseline and after six months of study medication. We will determine whether addition of letrozole alters the frequency of menopausal symptoms.

Adverse Events and Quality of Life – Change from baseline to six months for hot flash score (Mayo Clinic), fatigue (brief fatigue inventory), overall quality of life (BCPCT), bone turnover markers, HDL/total cholesterol, and C-reactive protein will be compared between the two groups using the two-sample t-test. Similarly, demographic variables (e.g., age, education) and hormonal levels will be tested for use as covariates on each of these endpoints. We will also assess the effects of change in serum hormones (if any) and baseline 25-OH vitamin D levels on incidence and severity of arthralgias and fatigue.

Within Letrozole Comparisons at baseline, 6 and 12 months - On all of our quantitative continuous measures we will perform a repeated measures analysis comparing the 6 and 12 month values to baseline and also with each other. This will give us some preliminary indication whether there is any benefit or detriment of 12 months of treatment. For all the above analyses of exploratory endpoints, as well as for changes in proliferation, results from the 12-month timepoint (following six months of open-label treatment with active agent) will also be analyzed and compared to the baseline and six month values with caution since the double-blind, randomized nature of the comparison is no longer present. However, since

every effort is made to conduct the assessment and quantification of biomarkers without knowledge of treatment arm, the results could be informative.

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

13.0 REGULATORY

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

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

Novartis and NCI, DCP as the funder of the study, have the right to request discontinuation of the study at any time. A frequency of greater than grade 2 adverse events (exclusive of hot flashes and menstrual irregularities) that exceeds 20% will be cause for consideration of stopping the trial; unless there is adequate information to suggest that the adverse events are not related to the study agent.

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