Title: 2017-073: The Effect of Statins on Markers of Breast Cancer Proliferation and Apoptosis in Women with Early Stage Breast Cancer

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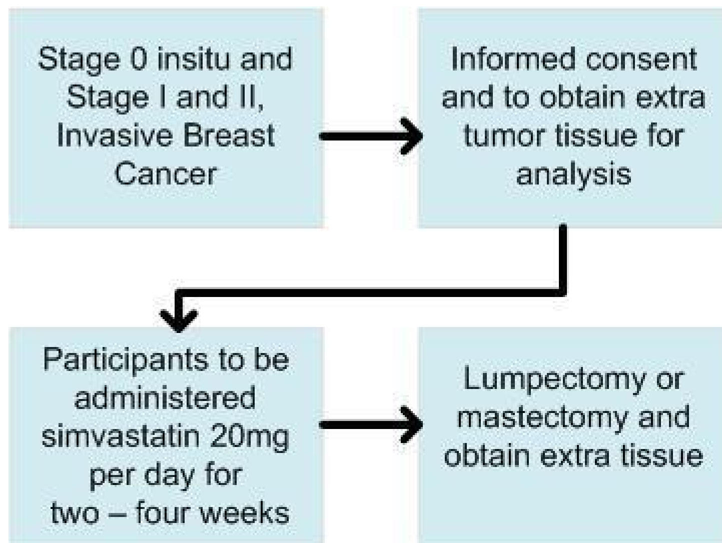
Protocol Synopsis

The purpose of this Window of Opportunity study is to identify the molecular and genetic mechanisms by which statins influence breast cancer cell proliferation. Preclinical data suggest that statins, the most commonly used cholesterol-lowering medication, may influence mammary cancer growth, but the clinical evidence is inconsistent. Epidemiologic studies suggest that prior statin intake may be associated with earlier stage breast cancer at diagnosis, lower risk of relapse and lower breast cancer mortality rates. Our study sample will include women with newly diagnosed clinical stage 0 (insitu) and stage I and II, invasive breast cancer who will be treated with simvastatin (20 mg) for 2 to 4 weeks in the window of time between their initial breast cancer diagnosis until the time of definitive breast surgery. Left-over breast tumor tissue from both the diagnostic and surgical specimens will be obtained for analysis. The primary study endpoint will be change in expression from pre- to post—treatment of tissue levels of ki-67, a marker of breast tumor proliferation. Secondary endpoints will include changes in other candidate predictive markers of breast tumor proliferation which will include cyclin D1 and P27; as well as changes in markers of apoptosis (cleaved caspase-3 (CC3)); inflammation (c-reactive protein (CRP)), composition of the plasma membrane (lipid rafts) as well as tumor-specific CYP27A1 expression. We will also measure changes in tumor signaling molecules that may be also altered by cholesterol depletion including estrogen receptor (ER), progesterone receptor (PR) and HER2neu as well as any changes in tumor grade. Since the primary effect of statins includes the inhibition of hydroxymethylglutaryl CoA reductase (HMG-CoA), we will evaluate the effect of statins on cell proliferation and apoptosis based on tumor expression of HMG- CoA. As a pilot analysis we will compare the effect of statins on breast tumor proliferation and apoptosis between groups defined by tumor expression of ER, PR status, HER2neu and histologic grade. This study will add to the field by confirming the results of two prior window of opportunity studies in similar patient populations. We will in addition, add new biomarkers previously not explored in this clinical situation including composition of the plasma membrane (lipid rafts) and activation of signaling markers (pAkt, pMAPK, pEGFR, pHER2).

Study Schema



Study Glossary

Definitions

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1) OBJECTIVES

a) Evaluate the relationship between short-term use of oral simvastatin on change in expression of Ki-67 as a candidate biomarker of breast tumor proliferation among women with clinical stage 0 insitu and 1 or 2- primary invasive breast cancer.

b) Evaluate the relationship between short-term use of oral simvastatin on changes in other candidate predictive markers of breast tumor proliferation (cyclin D1 and P27), changes in a marker of apoptosis (cleaved caspase-3 (CC3), changes in a marker of inflammation (c-reactive protein (CRP), novel additional biomarker changes in the composition of the plasma membrane (lipid rafts), changes in .activation of signaling markers (pAkt, pMAPK, pEGFR, PHER2) and changes in serum levels of 27-hydroxycholesterol (27OHC) and tumor expression of CYP27A1.

c) To conduct exploratory analyses comparing the effect of statins on breast tumor proliferation and apoptosis in groups defined by tumor expression of hydroxymethylglutaryl CoA reductase (HMG-CoA), ER/PR status, HER2neu, and tumor grade.

2) BACKGROUND AND RATIONALE

a. Introduction: Statins are a class of medications that are competitive inhibitors of Hydroxy Methyl Glutaryl Co-enzyme A (HMG-CoA) reductase which is the rate-limiting enzyme in the cholesterol bio-synthesis pathway. As a result, statins lower total (TC) and low density lipoprotein cholesterol (LDL-C) and have a favorable impact on cardiovascular mortality (1). The downstream effects of statins are not limited to inhibition of cholesterol synthesis alone. Statins have anti-inflammatory effects thought to be important in the setting of acute myocardial infarction which also may be a mechanism involved in anti-carcinogenic properties of statins (2). Furthermore, statin inhibition of the mevalonate pathway may impact Ras and RhoGTPases which are important in cell proliferation, migration and apoptosis and as such support a role for the anti-cancer effects of statins (3-6). All of the alterations associated with statins may play a role in the anti-cancer effect of statins.

Statins are chemically classified based on their solubility into octanol soluble (lipophilic) or water soluble (hydrophilic). Lipophilic statins (lovastatin, simvastatin, fluvastatin) penetrate the plasma membrane and are reported to interrupt mevalonate synthesis both in the liver and periphery while hydrophilic statins (pravastatin and atorvastatin) are taken up only by active transport and only affect selected hepatocytes that have transporter molecules (7) . Reports in the literature suggest a differential effect of statins on cancer prevention based on the type or class of statin (8-11). It is postulated that the cellular uptake of lipophilic statins may be related to their inhibition of cell growth. This concept is supported by a cell culture study in which only lipophilic statins were shown to have anti-cancer activity (10).

b. **<u>Epidemiologic Studies</u>**: Population based studies of the association between statins and cancer risk have demonstrated mixed results with some studies showing a protective effect for some cancers (12-15) and other studies showing an increased cancer risk or no association (16-19). Two meta-analyses evaluated the effect of statins on breast cancer risk, one of which included 7 randomized trials and 9 observational studies (20) and the other, 7 randomized trials (21) and both showed no significant association. A recent prospective cohort analysis showed no significant relationship between prior statin use and colon, lung, pancreatic, breast or bladder cancer however there was a reduction in risk of melanoma, endometrial cancer and NHL (22). Another nested case control study revealed an increased risk of bladder, colon and lung cancer associated with past statin use, however a reduced risk of hematological malignancies including myeloma, leukemia and lymphoma (23). In a meta-analysis of twenty case control studies, there was a protective effect for colon cancer, but no effect on breast, lung and prostate cancer risk (24). Other studies have demonstrated a protective effect of statins including a US veteran's study which demonstrated a reduced risk of pancreatic cancer (25) and three recently completed meta-analyses showed a reduced risk of colon cancer (26-28). While the results of epidemiological studies of statins and cancer risk are conflicting, other studies looking at the relationship between statins and breast cancer outcome suggest a possible protective effect showing a relationship between prior statins and earlier stage at diagnosis (29;30), lower rates of relapse (31-33) and lower breast cancer mortality (30;34;35).

c. **<u>The effect of statins on breast tumor proliferation, apoptosis and cell-cycle regulation</u>**: A number of preclinical findings support the biologic plausibility of a protective effect for statins in relationship to the development of breast cancer. Statins act by inhibition of HMG-CoA reductase (HMG-CoAR) and have pleomorphic properties in the cell. In-vitro data has shown anti-proliferative, apoptotic and anti-invasive properties of statins in cancer cell line studies (36-41). In vitro studies of breast and lung cancer cell lines have reported statin induced decrease in expression of anti-apoptotic BCL-2 and increase in pro-apoptotic BAX protein (42). It has been postulated that a change in the balance of pro-and anti-apoptotic signals toward the former may enhance the apoptotic activity of chemotherapy.

   Another possible chemo preventive activity of statins may be related to statin induced inhibition of the mevalonate pathway (5;38;43;44). Farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), downstream products of the mevalonate pathway, are both involved in posttranslational modification of many proteins such as the Ras molecule (45), which in turn helps transmit downstream signaling from surface receptors (46). Ras is involved in many intracellular pathways and increases gene transcription and proliferation by acting through the MEK and phosphoinositide 3-kinase (PI3K)/Akt pathways (36). Inhibition of this pathway can have anti-proliferative effects on cancer cells (37).

Statins have also been implicated in reducing cell migration, proliferation, and invasion by inhibiting production of GGPP, which is involved in geranylgeranylation of Rho proteins including Rho GTPases (38) that maintain function of Rho kinases involved in various cellular functions including gene expression, actin cytoskeleton migration, adhesion, and contractility of cells (47). Preclinical studies have demonstrated that statin treatment alters the composition of the plasma membrane. By reducing overall cholesterol concentrations, membrane structures referred to as lipid rafts are disassembled and key growth factor receptors are un-clustered. Several groups have demonstrated an increase in sensitivity to tyrosine kinases inhibitors, including EGFR and HER2 inhibitors, in the presence of statins (1-3).

In a pre-operative study of women with stage 0 and 1 breast cancer, fluvastatin (80 mg vs. 20 mg) was administered for 3-6 weeks between the time of initial diagnosis and up until the time of the definitive breast cancer surgery. Statin use was associated with a reduction in breast tumor proliferation (7.2% reduction in Ki-67 expression in high grade tumors vs 0.3% reduction in low grade tumors) and a preferential increase in tumor apoptosis in high-grade vs. low-grade tumors (60% vs. 13%) as well as ER-negative vs. ER-positive tumors (55% vs 28%) (48). In a similar study of atorvastatin 80mg administered for 2 weeks among women with stage I and II invasive breast cancer, there was a 7.6% reduction in Ki67 expression with a higher reduction of 68% noted among breast tumor samples that had high expression of HMGCoAR (49).

Other studies have focused on molecular explanations of the effect of statins In a study of murine xenografts, simvastatin stimulated production of a variant of the p53 transcription factor and diminished the potential to form bone metastases (50). Other evidence also implicates the p53 pathway. A 3-dimensional breast cancer cell culture model showed that mutant p53 up-regulated the mevalonate pathway to induce an invasive phenotype. Addition of simvastatin to the culture medium caused reversion to normal morphology, while the malignant phenotype persisted when both simvastatin and GGPP were present in the medium (51).

Borgquist etal suggested that the antiproliferative action of statins is due to an effect on cyclin D1 and p27, through up-regulated expression of the tumor suppressor p27 and down-regulated expression of the oncogene cyclin D1 in breast cancer. (52). In normal cells, progression through the cell cycle is controlled by the cyclin dependent kinases (CDKs), a family of serine/threonine kinases (53). The CDKs form complexes with their regulatory units, cyclins, thereby activating the CDKs, leading to phosphorylation of the cell cycle regulatory proteins that initiate and regulate progression through the different phases of the cell cycle (54). In breast cancer cells, the cell cycle control system is deregulated at multiple levels, leading to abnormal cell proliferation (55). Cyclin D1 is a vital regulator of the G1/S transition. The interaction of cyclin D1 with CDK4 and CDK6, leads to phosphorylation and thereby inactivation of the Rb-protein and its G1-maintaining

function, which culminates in the expression of proliferation-associated target genes (56;57). Cyclin D1 is overexpressed at the protein level in up to 50% of all primary breast cancers, in part due to amplification of the cyclin D1 gene, CCND1 (58). The CDK inhibitor p27, also known as Kip1, is involved in the regulation of the G0-to-S-phase transition. p27 interacts with CDK2-cyclin E, CDK4/6-cyclin D, and CDK2-cyclin A complexes, thereby strictly regulating these complexes (59;60). The tumor suppressor p27 is frequently deregulated in breast cancer, and reduced p27 expression has been associated with increased proliferation, high tumor grade, HER2 amplification as well as estrogen receptor (ER) and progesterone receptor (PR) negativity. Feldt and colleagues showed that following statin treatment that cyclin D1 expression was significantly decreased and that the protein expression of the tumor suppressor p27 increased significantly (52).

d. Impact of 27-hydroxycholesterol in breast cancer development: 27-hydroxycholesterol (27OHC), is the most prevalent oxysterol (38;39)(40), and is a primary metabolite of cholesterol. 27OHC is generated enzymatically by the P450 enzyme sterol 27-hydroxylase (CYP27A1) which is present in the liver, intestine, vasculature, brain as well as macrophages (39) and catabolized by 7-alpha hydroxylase CYP7B1, which is abundant in the liver (38;39;41). 27OHC functions as a naturally occurring ER ligand and acts as an in-vivo SERM that exhibits ER-agonist activity (42;43) demonstrated by its induction of a unique active conformation of ER alpha (42;43). Oxysterols also serve multiple roles in lipid metabolism (38;44), and function as ligands for the liver X receptor (LXR) alpha and beta (45). Results from cell culture and mouse models have led investigators to hypothesize that the relationship between obesity, high cholesterol and breast cancer is modulated through the action of endogenous 27OHC (24;40;46-48), and that endogenous production of 27OHC is in part responsible for acquired resistance to Tamoxifen (49) and AI's (36).

With the identification of 27OHC as an endogenous SERM, studies have evaluated its potential activity on ER positive breast cancer cell lines. In a series of experiments, 27OHC has been shown to upregulate ER alpha, increase cyclin D1 expression, increase the number of cells entering S phase, and increase overall cell number (50). Wu et al (51) and others (47) reported that 27-OHC stimulates MCF-7 cell xenograft growth in mice. Wu also showed that 27OHC is produced by MCF-7 cells, and stimulates cell-autonomous, ER-dependent and GDNF-RET-dependent cell proliferation concluding that 27OHC is a locally-modulated, non-aromatized ER ligand that promotes ER sensitive breast tumor growth. He also demonstrated that diminished expression of CYP7B1 (enzyme responsible for 27OHC metabolism) is associated with higher levels of 27OHC.

In another series of experiments, Nelson et al (24) demonstrated that 27OHC increases the growth of ER-positive breast cancer, and that the genetic or pharmacologic inhibition of 27OHC attenuates hypercholesterolemia-promoted tumor growth. Other mouse experiments demonstrated that elevated 27OHC was associated with increased frequency of lung metastases (24). In a series of studies using aggressive human breast cancer cell models, Nelson identified high levels of CYP27A1 (enzyme which converts cholesterol to 27OHC), suggesting that intra-tumoral 27OHC production may play a role in carcinogenesis. In a study of AI-resistant breast cancer cell lines, Nguyen found that active cholesterol biosynthesis sparks the activation of ER alpha, partly by the synthesis of 27OHC (36). Raza, et al investigated the extent to which 27OHC regulates cell proliferation in MCF7 - ER-positive breast cancer cell lines and found that treatment of MCF7 cells with 27OHC resulted in reduced p53 transcriptional activity, and was associated with increased levels of the E3 ubiquitin protein ligase MDM2, and decreased P53 levels (47).

In the only reported study of 27OHC in women with breast cancer, Wu et al (51) measured serum 27OHC in a sample of 66 breast cancer cases and 18 age and race-matched controls. While the results showed that mean 27OHC serum levels were similar for cases and controls (approximately 120 ng/ml), mean levels in 10 cases were more than 2SD's above that of controls, suggesting some variability among affected women with breast cancer. It is unclear whether the heterogeneity in these results reflects a true biologic difference possibly related to tumor burden, or whether the differences are only due to chance. In his study, Wu et al also compared levels of 27OHC in normal and tumor tissue and normal tissue in controls and demonstrated a 3-fold higher level of 27OHC in normal tissue from cases compared to controls with further enhancement of 27OHC in breast tumors. They found no observed association between serum and tumor 27OHC (51).

To evaluate the relationship between 27OHC and coronary heart disease (CHD), Rossouw et al, used a nested case-control design and archived serum samples from 350 women with CHD and 813 matched controls from the WHI Hormone Therapy trial (52). 27OHC levels were analyzed at the Atherosclerosis Research Lab at the Baylor College of Medicine, using a using a modified isotope-dilution gas chromatography-mass spectrometry method (53). There were no noted differences in 27OHC levels in cases vs. controls (36.5 nmol/L vs 37.7 respectively) however 27OHC was significantly correlated with high density lipoprotein cholesterol (HDLC), low density lipoprotein cholesterol (LDLC) and total cholesterol (TC). For each 10% increase in serum 27OHC, there was a significant 8% decrease in HDLC and a significant 14% and 18% increase in LDLC and TC, respectively. 27OHC was inversely correlated with BMI

in this study. Finally 27OHC was not independently associated with risk of CHD, nor did it modify the relationship between hormone therapy and CHD risk.

We have utilized Women's Health Initiative (WHI) data to study the relationship between statins and: cancer risk (54-58); breast cancer stage at diagnosis and mortality (59) and overall cancer mortality (60). Whereas our results suggest a very modest chemo-protective role for statins (54;56;58) we demonstrated that statins may be associated with better breast cancer survival (59), a result which has also been replicated by others (61-63). We hypothesize that one possible mechanism for the protective role of statins is through the reduction in circulating levels of cholesterol, resulting in lower levels of 27OHC and/or intratumoral levels of CYP27A1. If true, this would suggest that 27OHC may be a modifiable risk factor for breast cancer (40;49;64).

e. **Summary**: Epidemiologic and laboratory studies suggest that statins may have a favorable impact on clinical outcomes among women with breast cancer, however there have only been two published window of opportunity (perioperative) studies which have directly evaluated the effect of statins on markers of breast cancer proliferation, apoptosis and cell cycle regulation (48;49). We propose to further replicate the findings of these studies which suggest that a short course of pre-surgical statins may have a favorable impact on markers of breast cancer prognosis. We will also conduct exploratory analyses comparing the effects of statins on breast tumor proliferation stratified by tumor expression of HMG-CoA, ER/PR status, HER2neu and tumor grade. We will use a "window-of-opportunity" mechanism to explore the effects of statins on women with clinical stage 0 insitu and I or II early stage invasive breast cancer in the short period of time between initial diagnosis and definitive breast surgery. Statins are widely accepted as treatment for high cholesterol and treatment for atherosclerotic heart disease, and associated with minimal toxicity, and if shown to have a favorable effect on markers of breast tumor growth, have the potential to lower the risk of long-term breast cancer and cardiovascular morbidity and mortality.

3. EXPERIMENTAL PLAN

We will use a phase II "window of opportunity" design in which women with clinical stage 0 insitu or stage I or II invasive breast cancer will be enrolled during the time period between their definitive breast cancer diagnosis after core needle biopsy and the final surgical removal of their tumor. At the Karmanos Cancer Center (KCC), the majority of women with newly diagnosed stage 0 insitu and stage I and II invasive breast cancer are evaluated in the Walt Multidisciplinary Breast Clinic at the Detroit campus of the KCC or at the Weisberg cancer treatment center in Farmington Hills, MI. The majority of women with newly diagnosed breast cancer are scheduled for definitive

surgical resection within approximately 4 weeks of their initial diagnosis. All participants in this window of opportunity study will be provided a dose of simvastatin 20 mg for at least 2 weeks and no longer than 4 weeks in the interval between biopsy and definitive surgery. Simvastatin is one of the most commonly prescribed statins and is a lipophilic, high-potency statin, making it a reasonable candidate statin for this study. Simvastatin will be provided free of charge to the participants by the investigator through the KCI pharmacy. We will analyze both pre- and post-treatment biopsy/tumor specimens for markers of breast tumor proliferation, apoptosis, cholesterol metabolism, cell signaling and intra-tumoral expression of CYP27A1 and will analyze tumors as well for standard markers of breast cancer prognosis (ER, PR, HER2neu and tumor grade). This study will be conducted at the KCC (Walt Center and Weisberg and other Network Centers including Flint and Bay City) as well as Ascension Providence Hospital and the Henry Ford Health Systems as the clinical sites. As part of the protocol we will collect pre and post-surgical blood specimens to measure serum levels of total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and 27-hydroxycholesterol (27OHC). The pre-surgical blood specimen will be obtained at the screening visit and the post-surgical blood specimen will be collected up until the first post-operative visit.

4. SUBJECT ELIGIBILITY:

a. Inclusion Criteria

i. Provision of informed consent prior to any study specific procedures.
ii. Female patients must be > 18 years of age.
iii. Histologic confirmation of insitu or invasive breast cancer with any measures of ER, PR and HER2neu.
iv. Clinical stage 0, I or II breast cancer for which there will be at least a 2 week period of time between diagnosis and definitive surgery.
v. Performance Status (EGOG 0-2).
vi. Not currently pregnant during the study. Participants will be informed that the use of contraceptive pills is contraindicated because it may interfere with the study drug and it may be harmful to the woman who has been diagnosed with breast cancer.

b. Exclusion criteria

a. Plans for administration of neoadjuvant chemotherapy or hormonal therapy.
b. Previous or concurrent malignancy (with the exception of non-melanomatous skin cancer) within 1- year of the date of the consent.
c. Severe gastrointestinal disorder.
d. Current use of statins or fibrates for any time during the 3 months prior to the study.
e. Proven hypersensitivity to statins.

f. Mild or higher alterations of hematologic, liver and renal function. (i.e., WBC <2,500 /mm3, Plt <100,000/mm3, HgB <8 g/dL, AST >45 U/L, ALT >45 U/L, creatinin >1.5 mg/dL, bilirubin >1.15 mg/dL and CPK > 250 mg/dL);
g. CNS diseases and major psychiatric diseases or inability to comply to the protocol procedures;
h. Active infections;
i. Cardiac failure, class I-IV;
j. Current treatment with coumadin.
k. Mitral and/or tricuspid valvulopathy or valvular prosthesis; Angina; Severe arterial hypertension;
l. Current lactation.

5. SUBJECT ENROLLMENT

a. Enrollment of subjects: Subjects will be approached for enrollment at the time of the first visit with a breast surgeon after their definitive breast biopsy. Clinical documentation of the subject consent and enrollment will be included in the dictated note.

b. Breast tumor specimen collection:

i. Extra biopsy tissue blocks will be requested after subject registration, for IHC staining from the initial biopsy specimen.
ii. At the time of the lumpectomy or mastectomy, two additional portions of tumor not required for pathological diagnosis will be formalin fixed and paraffin embedded and flash frozen.

6. TREATMENT PROCEDURES

a. Simvastatin will be obtained from commercial sources and will be provided free of charge to the participant through the KCI pharmacy. Participants will take simvastatin 20 mg daily for at least 2 weeks and no longer than 4 weeks, from the time of consent to the day prior to their definitive breast surgery. A pill diary will be maintained through the clinical trials office.

b. Serum total cholesterol (TC), low-density-lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) will be measured in the KCI tissue core lab (PI Dr. Julie Boerner) at study entry, and at the end of statin administration. Pathology slides and tissue block material will be obtained from clinical pathologic material not needed for clinical purposes from the biopsy specimen and lumpectomy or mastectomy specimen.

c. Participants will be monitored for toxicity through telephone monitoring and clinic visits as needed. Telephone monitoring will occur 3-5 days after initiating simvastatin and within 2 weeks of stopping simvastatin. Details about reported side effects will be maintained by the study nurse.

d. Participants will be provided a $25 Target gift card after the completion of all study related activities.

7. STUDY PROCEDURES

a. CANDIDATE BIOMARKERS

i. Tissue Markers

1. Ki-67 is a marker of breast tumor proliferation and will be the primary marker of interest.
2. Other markers of breast tumor proliferation will include cyclin D1, and P27.
3. Cleaved caspase-3 (CC3) is a marker of apoptosis.
4. C-reactive protein (CRP) is a marker of inflammation.
5. Cholesterol metabolism (HMGCoAR)
6. Composition of the plasma membrane: Lipid rafts
7. Activation of signaling markers (pAkt, pMAPK, pEGFR, pHER2)
8. Intra-tumoral levels of CYP2A1
9. ER, PR and HER2neu (clinical pathology laboratory)

ii. Serum Markers

1. Total cholesterol (TC)
2. Low-density-lipoprotein cholesterol (LDL-C)
3. High-density-lipoprotein cholesterol (HDL-C)
4. Serum 27-hydroxycholesterol

b. LABORATORY METHODS

c.

i. Immunohistochemistry (IHC): IHC will be performed using well established protocols. Percent positive cells will be compared between the pre-surgical biopsy and the surgical resection using 3D Histotech software. Specifically, to assess the changes in tumor growth a standard marker of cellular proliferation, Ki67 will be utilized. Common markers of breast tumors that define treatment course will also be analyzed by IHC. Concentration and localization of ERα, PR, HER2 as well as intra-tumoral expression of CYP27A1 also will be assessed. To determine the concentration and localization of cholesterol in the tumor specimens, labeled-cholera-toxin B will be utilized. From these studies we expect to find that Ki67 will be reduced in the surgical specimen compared to the biopsy specimen. In addition, we would expect that the cholesterol detected in the cell would be reduced. If cholesterol is reduced, we would expect to see a differential activation of key signaling molecules including HER2, EGFR, Akt, and MAPK (if expressed).

ii. HMGCoAR activity will be measured in tumor specimens via an in vitro assay that assesses the ability of a specimen to reduce HMGCoA into mevalonate and coenzyme A. Protein will be isolated from flash-frozen tumor specimens and utilized as the source of enzyme for this biochemical assay. Readout for the assay is a

change in spectrometric counts over time. From these experiments, we expect for the first time, to see a reduction in HMGCoAR activity in the surgical tumor specimen when compared with the biopsy specimen.

iii. 27OHC: We will utilize the laboratory at the Baylor College of Medicine to assay 27OHC on serum using a modified isotope-dilution gas chromatography-mass spectrometry (GC-MS) method (52;52). Briefly, lipids will be extracted from the serum using a modified Folch technique. Oxysterols will be purified from other lipids by solid-phase extraction followed by derivatization and analysis by gas chromatography- mass spectrometry (GC-MS) on an Agilent 5973 (Agilent Technologies, Santa Clara, CA). In a prior analysis of 27OHC in the Women's Health Initiative, the inter-assay coefficient of variance was 16.6% and intraclass correlation of 55 blind duplicates was 0.47 (52). Analyses will be run in batches. Ten percent blinded duplicates will be included and between run coefficients of variation will be computed. We will use 250 ul aliquots from the archived KCI bio-repository for the analysis. uSerum levels of TC, LDL-C and HDL-C will be conducted in the laboratory of Dr. Julie Boener at the KCI.

8. REMOVAL AND REPLACEMENT OF SUBJECTS

In the absence of treatment delays due to adverse events, treatment will be completed unless the patient experiences any one of the following:

- A change from early stage to locally advanced or metastatic at the pre-surgical restaging.
- Inter-current illness that prevents further administration of the treatment.
- Unacceptable adverse event(s) including severe myopathy.
- The participant decides to withdraw from the study.
- General or specific changes in the patient's condition that render the patient unacceptable for further treatment in the judgment of the investigator.

9. SAFETY DATA COLLECTION, RECORDING, AND REPORTING

Data and Safety Monitoring

1. Scheduled research meetings will be held monthly or more frequently depending on the activity of the protocol. These meetings will include the protocol investigators and research staff involved with the conduct of the protocol.

2. During these meetings the investigators will discuss:
   - Safety of protocol participants (adverse events and reporting)
   - Validity and integrity of the data (data completeness on case report forms and complete source documentation)
   - Enrollment rate relative to expectation of target accrual, (eligible and ineligible participants)

- Retention of participants, adherence to the protocol and protocol deviations
- Protocol amendments

3. Data and Safety Monitoring Reports (DSMR) of the research meetings will be completed by the Study Coordinator and submitted to the Data and Safety Monitoring Committee monthly for review.

4. The Barbara Ann Karmanos Cancer Institute, Data and Safety Monitoring Committee (DSMC) provides the primary oversight of data and safety monitoring for KCI Investigator-initiated trials.

Data Management

Clinical trial data will be captured in OnCore (Clinical Trial Management System) electronic data capture system.

Trial data entered in Oncore will be verified by source documentation. The eCRFs should be completed by within 10 business days of the availability of clinical documentation of a study visit. Data clarification and queries should be completed within 7 working days of notification.

A KCI CTO QA Monitor will monitor essential clinical trial data.

10. ADVERSE EVENTS

The most commonly reported adverse reactions (incidence ≥ 5%) in simvastatin controlled clinical trials are upper respiratory infections, headache, abdominal pain, constipation, flatulence, nausea, vomiting, dyspepsia and asthenia. Other side effects reported are diabetes mellitus, cognitive impairment, pruritus, alopecia, a variety of skin changes (e.g., nodules, discoloration, dryness of skin/mucous membranes, changes to hair/nails), dizziness, muscle cramps, myalgia, arthralgia , pancreatitis, paresthesia, peripheral neuropathy, anemia, erectile dysfunction, interstitial lung disease, hepatitis/jaundice, fatal and non-fatal hepatic failure and depression .

The incidence of myopathy, including rhabdomyolysis was < 0.1%, myopathy (defined as unexplained muscle weakness or pain with a serum creatine kinase [CK] > 10 times upper limit of normal [ULN]). Rhabdomyolysis (defined as myopathy with a CK > 40 times ULN) is rare. Rarely hypersensitivity reactions are noted.

Drug interactions:

Concomitant use of CYP3A4 inhibitors, cyclosporine, danazol is contraindicated. If treatment with itraconazole, ketoconazole, posaconazole, voriconazole, erythromycin, clarithromycin is unavoidable, therapy with simvastatin must be suspended during that course. The risk of myopathy, including rhabdomyolysis is increased by concomitant

administration of cyclosporine or danazol, amiodarone, dronedarone, ranolazine, or calcium channel blockers such as verapamil, diltiazem, or amlodipine, colchicine, and gemfibrozil. Protease inhibitors, such as saquinavir and ritonavir increases pancreatitis risk. Concomitant administration of digoxin with simvastatin resulted in a slight elevation in digoxin concentrations in plasma. Patients taking digoxin should be monitored appropriately when simvastatin is initiated.

11. STATISTICAL CONSIDERATIONS

Objectives: The primary objective of this study is to assess, in women with clinical stage 0 insitu and stage stage I-II invasive breast cancer, the effect of simvastatin on a biomarker of proliferation, Ki-67. The secondary objectives are to assess the effect of simvastatin on biomarkers of apoptosis, inflammation, lipid rafts and other biomarkers. The tertiary objectives are to evaluate differences in Ki-67 between groups defined by tumor expression of hydroxymethylglutaryl CoA reductase (HMG-CoA), ER/PR status, HER2neu, and tumor grade.

Study Design: This study has a repeated measures study design. Biomarkers will be assessed just before initiation of statin treatment and again just before surgery.

Endpoints: The primary endpoint is change in Ki-67 expression, a marker of breast tumor proliferation, expressed as a change in the percentage of KI-67 positive cells.

Exploratory endpoints include changes in other candidate markers associated with breast tumor proliferation including cyclin D1 and P27, cleaved caspase-3 (CC3) as a marker of apoptosis, and c-reactive protein (CRP) as a marker of inflammation. These biomarkers are measured on a 5-point ordinal scale according to the proportion of cells stained, negative (0-1%), weak (2-20%), moderate (11-50%), strong (51-75%), or very strong (>75%).

Statistical Analysis Plan: Before beginning the analysis, the shape of the distribution of change in percentage of Ki-67 positive cells will be assessed and a transformation applied if needed to meet the statistical assumptions. Changes in Ki-67 will be described as average pre-post differences in percent positive cells with 95% Wilson confidence intervals and tested with a paired t-test.

If the 5x5 pre-post data tables for the IHC measures of the other biomarkers are sparse, e.g. expected value of any cell < 5, categories will be collapsed. Statistical significance of changes in each of the other biomarkers will be assessed with a Wilxcoxon signed rank test.

Differences in changes between groups defined by each of the following lipid raft (+/), ER (+/-), PR (+/-), HER2neu (+/-), P53 (+/-) will be described with 95% confidence intervals and statistical significance will be assessed with Kruskal-Wallis tests.

Statistical Power and Sample Size: Sample size for this pilot study was determined by issues of feasibility. It is estimated that there may be insufficient tissue sample on as many as 10 participants (either at diagnosis or definitive surgery); therefore, we will plan to accrue a total of 50 participants.

Although numerous statistical tests and estimation procedures are planned, it is desired to guard against inflated type I errors. In order to provide some protection, the sample size calculations below assumed type I error of 0.01.

In pilot data on 20 women with early stage breast cancer from our institution, mean percent positive Ki-67 was 11.0% with a standard deviation (SD) of 12%. If all 50 women have adequate tissue, we will be able to detect a 6 percentage point decrease in Ki-67 if the correlation between pre and post KI-67 is moderate, with 85% statistical power, testing with 1% type I error (Table 1), If the dropout rate is 10% and the pre-post correlation is lower, then we will be able to detect a 9 percentage point decrease in Ki-67. Differences of that magnitude correspond to a moderate effect size and are likely to be of clinical importance.

Table 1. Detectable Pre/Post Differences
Assuming Mean Baseline Percent Positive Ki-67 = 11%, SD = 12%
Statistical Power = 85%, Two-sided Type I Error = 1%

N	Effect Size	Detectable Pre/Post Difference	Pre/Post Correlation
50	-0.53	-8	0.2
50	-0.53	-6	0.5
50	-0.53	-4	0.8
45	-.56	-8	0.2
45	-.56	-7	0.5
45	-.56	-4	0.8
40	-.60	-9	0.2
40	-.60	-7	0.5
40	-.60	-5	0.8

Detectable differences were calculated under the same assumptions for comparison of pre/post Ki-67 differences between groups defined by expression of hydroxymethylglutaryl CoA reductase (HMG-CoA), ER/PR status, HER2neu, and tumor grade. There will be 85% statistical power to detect large differences using Kruskal Wallis tests.

Table 2. Detectable Effect Size for
Comparison of Groups Defined by Biomarkers and Group Size

	Number in each group		
Power	12/28	16/24	20/20
80%	---	1.2	1.2
85%	1.4	1.4	1.4
90%	1.5	1.5	1.5

The predicted accrual rate for the study will be 2 participants per month. To account for potential slow accrual over holidays we will plan to complete study procedures within 2 years of opening the study.

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