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A Phase II Pilot Trial of Hydroxychloroquine, Everolimus or the Combination for Prevention of Recurrent Breast Cancer (“CLEVER”)

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List of abbreviations

HCQ: Hydroxychloroquine
RCC: renal cell carcinoma
AV: autophagic vesicles
mTOR: mammalian target of rapamycin
PFS: progression-free survival
CTEP: Cancer Therapy and Evaluation Program
PK: Pharmacokinetic
PD: Phramocdynamic
ASCO: American Society of Clinical Oncology
GBM: Glioblastoma multiforme
PI3K: phosphatidyl inositol 3-phosphate kinase
AUC: Area under the curve
VEGFR: vascular endothelial growth factor receptor
CQ: chloroquine
DLT: Dose limiting toxicity
MTD: Maximal Tolerated Dose
EM: Electron Microscopy
ECOG: Eastern Cooperative Oncology Group
ANC: Absolute neutrophil count
ALT: alanine transaminase
AST: Aspartate aminotrasnferase
RECIST: Response Evaluation Criteria in Solid Tumors
INR: International Normalized ratio
PTT: Partial thromboplastin time
HIV: Human Immunodeficiency virus
HBV: Hepatitis B Virus
HCV: Hepatitis C Virus
NCI: National Cancer Institute
CTCAE: Common Terminology Criteria for Adverse Events
AE: Adverse Event
CT: Computed tomography
DLCO: Diffusion capacity of the lung for carbon monoxide
HBsAg: Hepatitis B surface antigen
HBsAb: Hepatitis B surface antibody
HBcAb: Hepatitis B core antibody
DNA: deoxyribonucleic acid
RNA: ribonucleic acid
MRI: Magnetic resonance imaging
ECG: electrocardiogram
H&E: hematoxylin and eosin
IHC: immunohistochemistry
PBMC: peripheral blood mononuclear cell
PBS: phosphate buffered saline

IND: investigational new drug
IRB: institutional review board
FDA: federal drug administration
SAE: significant adverse event
DSMC: data safety and monitoring committee
CTRMSC: Clinical trial review and monitoring scientific committee
HIPAA: Health Insurance Portability and Accountability Act
CRF: Case report form

1 STUDY SUMMARY

Title	A Phase II Pilot Trial of Hydroxychloroquine, Everolimus or the Combination for Prevention of Recurrent Breast Cancer
Short Title	CLEVER Pilot Trial
Protocol Number	*IRB #826253; UPCC 24116*
Methodology	This is a randomized, controlled, open label pilot trial. A total of 60 patients will be enrolled, with 15 patients allocated to each treatment arm. Patients will be evaluated at the initiation of every cycle for adverse events, and dose reduction will be employed for toxicity. Bone marrow aspiration will be performed at the end of cycle 3, cycle 6, and if patient continued on second treatment phase, at the end of cycle 12. The initial six-month study period is designed to assess the effects of HCQ, EVE and their combination. After three months of observation, control patients will be offered HCQ/EVE investigational therapy for six cycles; thus, the control group is actually a delayed treatment group and all patients will receive treatment. Patients who continue to demonstrate positive bone marrow DTCs after six cycles will be offered the opportunity to continue on combination therapy for an additional 6 cycles. Patients will continue to be followed for recurrence events for up to 3 years after completion of treatment.
Study Duration	Active Enrollment: 3 years; Follow-up: 3 years
Study Center(s)	The University of Pennsylvania and affiliated sites
Objectives	**Primary Clinical Objective: To evaluate the feasibility of administering HCQ, EVE or the combination in patients who have completed primary therapy for breast cancer and harbor bone marrow disseminated tumor cells** ***Endpoint:*** $\geq$75% of patients in each arm reaching the 6-month evaluation time point without grade 3 or 4 toxicity. **Secondary Clinical Objectives:** **1. To determine the safety of administering HCQ + EVE in combination with adjuvant endocrine therapy** ***Endpoints*** Incidence of adverse events within each treatment stratum and dose level by NCI CTCAE v4 criteria. **2. To evaluate preliminary efficacy of HCQ, EVE or the combination in reducing or eliminating DTCs** ***Endpoint***

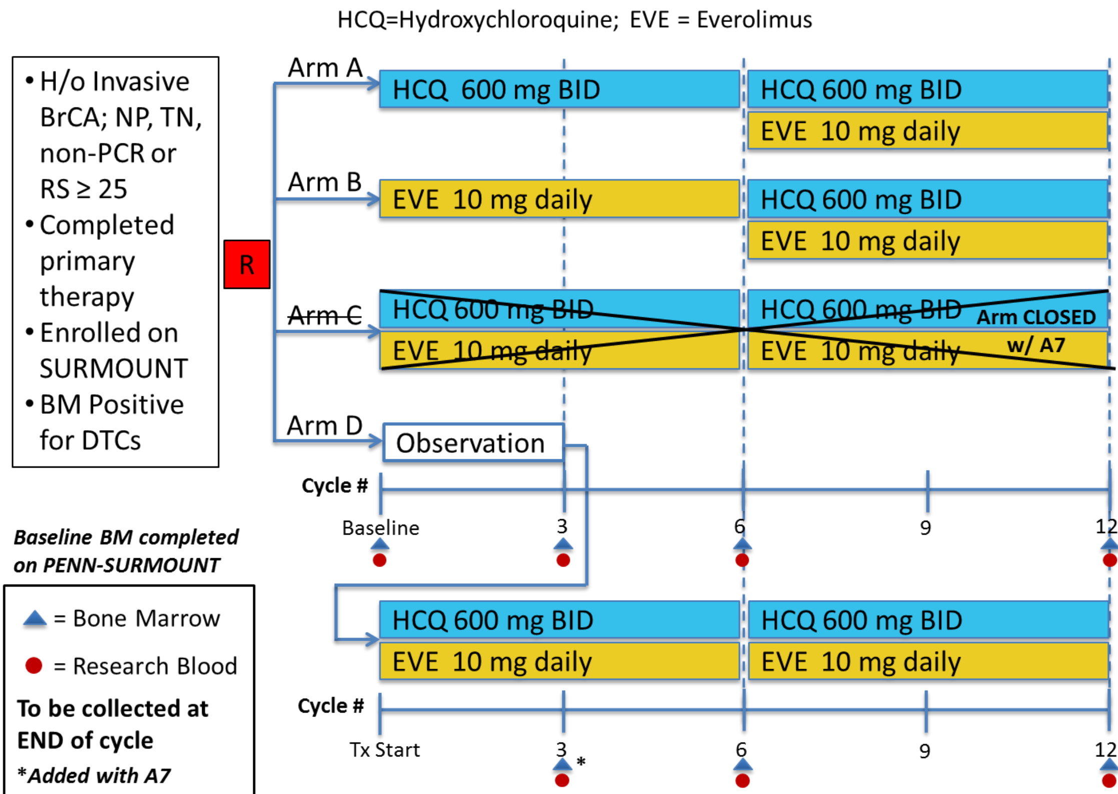
	• Percent change in bone marrow DTCs at 6 and 12 months of treatment relative to baseline (pre-treatment) • Incremental benefit of an additional 6 months of therapy in patients who have DTCs after initial 6 months of therapy • Incremental benefit of combination therapy compared to single-agent therapy **3. To estimate the risk of recurrence after treatment with HCQ, EVE or the combination** ***Endpoint*** • 3-year recurrence free survival (RFS) **Translational Objectives:** **1. To assess the utility of a novel DTC assay, DTC-Flow, as a pharmacodynamic marker in trial participants to detect DTCs and their response to study therapy, compared to DTC-IHC.** ***Endpoints:*** • Measurement of the number and type of DTCs by DTC-FLOW before, during and at the conclusion of study therapy • Comparison to measurement of DTCs by IHC **2. To determine whether patient DTCs biologically reflect primary tumor and predict response to study therapy.** ***Endpoints:*** • WES, RNA-seq, and CNV analysis will be performed on primary breast tumors and DTCs isolated by DTC-Flow to: • Identify similarities in molecular features between primary tumors and DTCs • Define genomic markers in DTCs that predict response to HCQ, EVE or their combination; 3) Identify similarities in molecular features between DTCs, CTCs and ptDNA. **3. To determine whether patient circulating tumor cells (CTCs) biologically reflect primary tumor, accurately identify patients with bone marrow DTCs at baseline, predict for the elimination of DTCs with therapy, and/or predict for improved clinical outcomes relative to therapy** ***Endpoints***: • Associations between biological and genomic features of

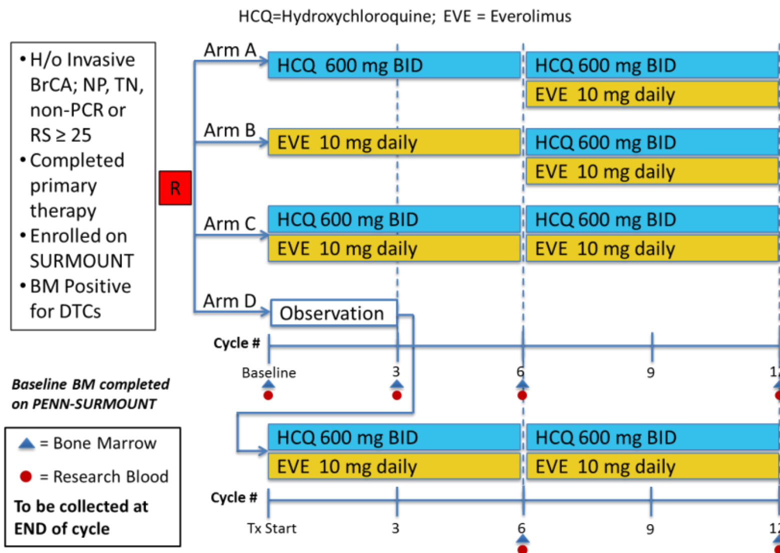
	CTCs and primary tumor • Measurement of CTCs before, during and at the conclusion of study therapy, compared to DTC and clinical outcomes. **4. To determine whether plasma tumor DNA (ptDNA) biologically reflects primary tumor, accurately identifies patients with bone marrow DTCs, or predicts response to therapy** ***Endpoints:*** Concordance of mutations between ptDNA and primary tumor Measurement of ptDNA before, during and at the conclusion of study therapy, compared to DTC outcomes.
Number of Subjects	The target number of study participants is 60
Diagnosis and Main Inclusion Criteria	• Histologically-confirmed, primary, invasive breast cancer diagnosed within 5 years of study entry. • Qualifying risk status, at diagnosis, meeting one of the following: o Histologically positive axillary lymph nodes o Primary tumor that is ER/PR<10%/Her2 negative o Primary tumor with Breast Cancer Recurrence Score of ≥ 25 per the Genomic Health Oncotype DX breast cancer test and/or high risk MammaPrint o Evidence of residual disease in the breast on pathological assessment after neoadjuvant chemotherapy. • Patients must have completed all primary therapy (definitive surgery, (neo)adjuvant chemotherapy adjuvant radiation and/or Her2-directed therapy) for the index malignancy at least 4 weeks prior to study entry. All prior treatment-related toxicity must be resolved prior to study enrollment. Concurrent receipt

	of adjuvant endocrine and bone modifying agents is allowed per standard of care guidelines. • Bone marrow aspirate after completion of therapy demonstrates detectable DTCs (via IHC) • No evidence of recurrent local or distant breast cancer by physical examination, blood tests (CBC, LFTs, Alk Phos), or symptom-directed imaging, per NCCN guidelines. • Age ≥ 18 years • ECOG performance status ≤ 2 • No contraindications to the study medications or uncontrolled medical illness. • Adequate bone marrow function as shown by: ANC ≥ 1.5 x 10^9/L, Platelets ≥ 100 x 10^9/L, Hb >9 g/dL • Adequate liver function as shown by: Serum bilirubin ≤ 1.5 x ULN, ALT and AST ≤ 2.5 x ULN, and INR ≤1.5 • Anticoagulation is allowed if target INR ≤ 1.5 on a stable dose of warfarin or on a stable dose of anticoagulant for >2 weeks at time of randomization. For patients on therapeutic anti-coagulants, medication must be clinically held peri-procedure (bone marrow aspirate) per standard clinical management. • Adequate renal function: serum creatinine ≤ 2.0 x ULN or creatinine clearance (CrCl) ≥ 30mL/min obtained within 28 days prior to registration. A calculated creatinine clearance by Cockcroft-Gault Formula is acceptable in lieu of a measured value. • Serum cholesterol ≤300 mg/dL OR ≤7.75 mmol/L AND triglycerides ≤ 2.5 x ULN. NOTE: In case one or both of these thresholds are exceeded, a fasting lipid assessment will be ordered. If one or both of these thresholds are still exceeded in a fasting state, the patient can only be included after initiation of appropriate lipid lowering medication. • Ability to provide informed consent
Statistical Methodology	The primary endpoint of this pilot study is feasibility. The secondary endpoints are tolerability and the measured effect of the investigational therapy on DTCs in patients with measureable DTCs at baseline and the comparison of this effect to that in a control population. Because this is proof-of-concept, sample sizes are not sufficient to address long-term recurrence risk. Randomization is being performed to minimize uncontrolled confounding. The trial endpoint “feasibility” will be defined as at least 11 of 15 patients within each treatment regimen completing 6 cycles of therapy without grade 3 or 4 toxicity. While arms may be

	discontinued for lack of feasibility, the trial may continue enrolling to those arms that remain feasible. Toxicity will be assessed using NCI CTCAE v4 criteria. Patients who drop out before completing cycle 6 for reasons other than grade 3/4 toxicity will be replaced. With 15 patients per group, an exact 95% confidence interval around the feasibility and toxicity rates will be no more than 0.52 units wide. We will define “response” for each patient as a 50% or greater decrease in DTC number at each treatment time point compared to baseline. A treatment arm/regimen will be considered successful if at least 3 of 15 of patients experience this predefined response endpoint. We will also estimate, within each treatment group, the % change in DTC number. While the trial is powered for feasibility, rather than detection of change in DTCs across treatment group, 15 patients per group will provide 80% power (using a two-group t-test with a two-sided alpha level of 0.05) to detect a decrease of approximately 1 standard deviation and will provide important preliminary data for designing future confirmatory trials.

1.1 Study Schema





2 STUDY OBJECTIVES

2.1 Primary Clinical Objective: To evaluate the feasibility of administering HCQ, EVE or the combination in patients who have completed primary therapy for breast cancer and harbor bone marrow disseminated tumor cells

Endpoint:

- ≥75% of patients overall completing 6 cycles of therapy without grade 3 or 4 toxicity

2.2 Secondary Clinical Objectives

2.2.1 To determine the safety of administering HCQ + EVE in combination with adjuvant endocrine therapy

Endpoints

- Incidence of adverse events within each treatment stratum and dose level by NCI CTCAE v4 criteria.

2.2.2 To evaluate preliminary efficacy of HCQ, EVE or the combination in reducing or eliminating DTCs

Endpoints

- Change in bone marrow DTCs at 3, 6 and 12 months of treatment relative to baseline (pre-treatment)
- Change in bone marrow DTCs at 3 months with no treatment (Arm D Control) compared to any treatment (Arms A, B or C)
- Incremental benefit of an additional 6 months of therapy in patients who have DTCs after initial 6 months of therapy
- Incremental benefit of combination therapy compared to single-agent therapy

2.2.3 To estimate the risk of recurrence after treatment with HCQ, EVE, or the combination

Endpoint

- 3-year recurrence free survival (RFS)

2.3 Translational Objectives

2.3.1 To assess the utility of a novel DTC assay, DTC-Flow, as a pharmacodynamic marker in trial participants to detect DTCs and their response to study therapy, compared to DTC-IHC.

Endpoints:

- Measurement of the number and type of DTCs by DTC-FLOW before, during and at the conclusion of study therapy
- Comparison to measurement of DTCs by IHC

2.3.2 To determine whether patient DTCs biologically reflect primary tumor and

predict response to study therapy.

Endpoints:

WES, RNA-seq, and CNV analysis will be performed on primary breast tumors and DTCs isolated by DTC-Flow post-NAC to:

- Identify similarities in molecular features between primary tumors and DTCs
- Define genomic markers in DTCs that predict response to HCQ, EVE or their combination; 3) Identify similarities in molecular features between DTCs, CTCs and ptDNA.

2.3.3 To determine whether patient circulating tumor cells (CTCs) biologically reflect primary tumor, accurately identify patients with bone marrow DTCs at baseline, predict for the elimination of DTCs with therapy, and/or predict for improved clinical outcomes relative to therapy

Endpoints:

- Associations between biological and genomic features of CTCs and primary tumor
- Measurement of CTCs before, during and at the conclusion of study therapy, compared to DTC and clinical outcomes.

2.3.4 To determine whether plasma tumor DNA (ptDNA) biologically reflects primary tumor, accurately identifies patients with bone marrow DTCs, or predicts response to therapy

Endpoints:

- Concordance of mutations between ptDNA and primary tumor
- Measurement of ptDNA before, during and at the conclusion of study therapy, compared to DTC outcomes.

3 BACKGROUND

Historically, cancer therapy has focused on drug targets found in the primary tumor. In recent years, however, it has become increasingly clear that the molecular pathways underlying breast cancer dormancy and recurrence are distinct from those of primary tumorigenesis. Consistent with this, our investigations in preclinical models indicate that dormant tumor cells possess unique vulnerabilities not observed in actively growing tumor cells. These unique attributes present a wealth of new drug targets that could hold the key to more effective treatments for this disease by preventing tumor recurrence and its associated mortality.

3.1 Disseminated tumor cells as a prognostic marker and target for therapy

Disseminated tumor cells (DTCs) can be detected in up to 30–40% of patients with early stage breast cancer(1, 2). A growing body of evidence has established that the presence of DTCs in these patients is an independent prognostic factor for recurrence(1, 3-5). Some of the strongest evidence for the prognostic significance of DTCs in breast cancer patients emerged from a pooled analysis of 4,703 patients with

stage I, II, or III breast cancer in whom DTCs were identified in 31% of patients(6). The presence of DTCs in bone marrow predicted poor breast cancer-specific survival, poor overall survival, and a high risk of loco-regional and distant recurrence. Additional studies, also using an immunohistochemical (IHC) assays to identify cytokeratin-positive (CK+) cells in bone marrow aspirates, have confirmed this association(7-9). Despite variability in the patient populations, assay methods, and antibodies used to identify DTCs in these studies, there is striking overall consistency in the rate at which DTCs were found and their prognostic significance, suggesting that DTCs are a powerful biomarker to identify early stage patients at significant risk of recurrence.

Despite mounting evidence for DTCs as a prognostic biomarker for recurrence, remarkably little has been done to utilize the presence of these cells either to identify patients at risk or as a target for adjuvant therapy. Currently, DTC assays are not used as standard of care, and other approaches to monitor for recurrence such as radiologic scanning and circulating tumor markers (e.g., CA27-29, CA15-3) are inaccurate and currently not recommended by the American Society of Clinical Oncology for use in the surveillance period(10). This lack of an active approach to monitor for residual cancer cells at a point where intervention could be successful leads to enormous distress for patients(11-13) and limits approaches to prevent incurable metastatic disease. This, in turn, has led to a paucity of data on whether targeting and eliminating DTCs would have a meaningful impact on recurrence and survival outcomes. Only one published study to date(14) has utilized the persistence of DTCs as an indication for additional adjuvant therapy. In this trial, patients who had completed 6 cycles of anthracycline-based adjuvant chemotherapy were tested for the presence of DTCs by IHC assay at 8 months post-treatment. Patients with at least 1 DTC detected were given 6 cycles of docetaxel. There was no randomized control group. Patients with persistence of DTCs post-docetaxel (21%) had significantly reduced disease-free interval (DFI) compared to those who had clearance of DTCs (HR 7.58, 95% CI 2.3-24.7). Patients with clearance of DTCs after docetaxel had comparable DFI to those who had no DTCs after anthracycline-based therapy alone. Though limited by the lack of randomization for treatment amongst those with DTCs, these important data suggest that clearance of DTCs may represent an effective strategy for reducing recurrence.

Building upon these data using therapies that specifically target unique features of DTCs represents a potentially transformative, innovative new approach to clinical trials that will reduce recurrence, as shown in the conceptual model in Figure 1. We now propose to conduct human studies that will translate to the clinic our findings in mouse models identifying unique biologic features of dormant minimal residual disease (MRD), including reliance for survival on autophagy and the mTOR pathway, and demonstrating that targeted inhibition of these pathways reduces the population of dormant residual tumor cells and improves recurrence-free survival (RFS). Key components of this effort are: 1) Novel clinical trials of agents targeting dormancy that are safe and tolerable in patients who, though harboring DTCs, may still be cured of their disease; and 2) Further refinement of the therapeutic approach through additional mouse preclinical studies and development of a flow-based DTC assay that will enable molecular and genetic characterization of both mouse and human DTCs to elucidate the biology of human tumor dormancy, identify appropriate patients and agents for intervention, and provide a pharmacodynamic marker of treatment effect. This approach, if successful, will enable real-time surveillance and intervention for patients at risk of recurrence, provide effective tools for proactive secondary prevention, and improve survival outcomes for patients with breast cancer.

PEN

3.2 Biology of tumor dormancy and recurrence

Dormant residual cancer cells likely constitute the reservoir from which recurrent tumors arise. Accordingly, the lack of biological understanding and therapeutic approaches aimed at these cells constitute major obstacles to the successful treatment of this disease. To address this critical gap, our laboratory developed a series of conditional genetically engineered mouse (GEM) models for breast cancer that faithfully recapitulate key stages of breast cancer progression, including tumor dormancy and recurrence, and phenocopy human breast cancers with respect to the oncogenic pathways activated (e.g., HER2, MYC, Wnt1, Akt), histological appearance, and tumor progression over time(15-24). Such models can facilitate the *in vivo* analysis of breast cancer progression and provide a platform for assessing the efficacy of interventions designed to prevent recurrent disease. Using these models, we found that virtually all mice bearing fully regressed tumors harbor dormant residual cancer cells in all common recurrent organ sites observed in patients, long after the apparently complete regression of their tumors. Furthermore, mice relapse at both local and distant sites with overt metastases. Accordingly, these models enable identification and functional analysis of the population of dormant residual cells that give rise to recurrent tumors, thereby permitting elucidation of the mechanisms underlying the persistence of minimal

residual disease and relapse.

3.2.1 GEM models recapitulate breast cancer dormancy and recurrence

The GEM models we have developed permit the inducible expression of oncogenes in the mammary epithelium of transgenic mice treated with doxycycline(15-24). Expression of the reverse tetracycline-inducible transactivator, rtTA, is driven in the mammary epithelium by the mouse mammary tumor virus (*MMTV*) LTR in mice bearing the *MMTV-rtTA* (*MTB*) transgene. Target transgenes are placed under the control of a *tetO* regulatory cassette. Transgene expression in this system is mammary-specific, rapidly inducible and deinducible, undetectable in the uninduced state, and can be titrated over a broad range(15-24). Using this system, we conditionally expressed the HER2/neu (*MTB/TAN*), c-MYC (*MTB/TOM*), Wnt1 (*MTB/TWNT*) and Akt (*MTB/TAKT*) oncogenes because of their demonstrated relevance to human breast cancer. Following oncogene induction with doxycycline, *MTB/TAN*, *MTB/TOM*, *MTB/TWNT* and *MTB/TAKT* bitransgenic (*MTB/Tet-Onc*) mice develop epithelial hyperplasias, focal atypical hyperplasias, and invasive mammary adenocarcinomas that spontaneously metastasize to the bone marrow, lungs, liver, lymphatics, , and brain – sites that are characteristically found in patients with breast cancer(25-28). Tumor formation is highly penetrant, mammary-specific, and entirely dependent upon transgene induction by doxycycline[15, 19].

3.2.2 Regressed tumors recur spontaneously in GEM

Strikingly, nearly all mammary tumors in *MTB/Tet-Onc* mice rapidly regress to a non-palpable state following oncogene down-regulation induced by doxycycline withdrawal, indicating that the vast majority of cells within these tumors are oncogene "addicted"(16, 18, 19, 24). The marked regression of primary tumors following oncogene down-regulation is similar to that observed in human cancer patients treated with targeted therapies, including trastuzumab(29, 30). However, breast cancer patients treated with chemotherapy, targeted therapies, and/or hormonal therapies often recur. As such, we suspected that at least some tumor cells would survive, and eventually resume growth, following oncogene down-regulation. To test this possibility, *MTB/Tet-Onc* mice in which doxycycline withdrawal had led to complete regression of primary tumors were monitored for tumor recurrence in the absence of doxycycline treatment. This revealed that ~80% of tumors recurred at their original sites over periods of up to one year, and that mice relapsed at distant sites as well(16, 18, 19, 31). Mutations detected in primary tumors were also present in recurrent tumors, indicating that they are *bona fide* recurrences(16, 18, 19). Tumor regression and recurrence occurs with similar kinetics in recipient mice bearing orthotopic primary mammary tumors derived from cultured or uncultured primary tumor cells from *MTB/TAN* or *MTB/TWNT* mice(16, 32-34). Further, we have observed a similar pattern of tumor regression and recurrence in mice treated with doxorubicin, cyclophosphamide and paclitaxel – chemotherapeutic agents that are commonly used to treat breast cancer patients(33).

These findings suggested that mice bearing fully regressed tumors harbor minimal residual disease in which growth can spontaneously reactivate, resulting in cancer recurrence. To test this hypothesis directly, *MTB/TAN* mice bearing fully

regressed tumors that had not recurred spontaneously within 6 months of HER2/neu down-regulation were reinduced with doxycycline and monitored for tumors. All mice rapidly re-developed tumors at their original sites within 1-2 wk(23). This demonstrates that, despite their clinical regression, all mice bearing regressed tumors harbor residual tumor cells in which growth can be reactivated.

Notably, in *MTB/TAN* mice maintained off doxycycline for extended periods of time following primary tumor regression, spontaneous recurrences arose with stochastic kinetics(20). Moreover, recurrent tumors grew at comparable rates regardless of whether they arose relatively early (i.e., 50 days) or late (i.e., 200-300 days) following tumor regression. Similar recurrent tumor growth rates for early or late relapses is also observed in breast cancer patients and is not compatible with a constant growth model for surviving residual tumor cells. Rather, this suggests that residual tumor cells may exist in a dormant state prior to recurrent tumor outgrowth.

3.2.3 Regressed tumors harbor residual neoplastic cells that are dormant.

A particularly valuable aspect of this model is the ability to identify and analyze residual tumor cells, which in turn enables elucidation of the mechanisms by which they survive, as well as determination of the impact of therapeutic interventions on this critical reservoir of cells. By comparing previously tumor-bearing and non-tumor-bearing mammary glands from mice harboring fully regressed tumors, we identified a histopathological lesion that is found only in glands that previously harbored a mammary tumor. Morphologically identical residual lesions were generated by orthotopically injecting cultured primary tumor cells from *MTB/TAN* mice into wild-type recipients maintained on doxycycline, allowing tumors to form, and then abrogating oncogene expression by doxycycline withdrawal. Furthermore, orthotopic residual lesions formed from GFP-labeled tumor cells, or the use of cre-activated ROSA-YFP reporter mice in combination with GEM tumor models, permit visualization of residual tumor cells in intact mice following tumor regression as well as their unambiguous identification, isolation and analysis.

To address directly whether residual tumor cells exist in a state of cellular dormancy, we performed BrdU labeling on tumor cells either within primary tumors in *MTB/Tet-Onc* mice maintained on doxycycline, or in residual lesions generated by oncogene down-regulation in tumor-bearing mice. While primary tumors exhibited high rates of proliferation, tumor cells within residual lesions were quiescent and exhibited lower proliferation rates than those observed in the adult mammary gland, which is itself a relatively quiescent tissue.

The concept of tumor dormancy implies that cells are capable of being “re-awakened”, whereby they reactivate growth. This contrasts with other possible explanations for low proliferation rates in residual tumor cells, such as terminal differentiation or senescence(35). To determine whether non-proliferating residual tumor cells are capable of re-entering the cell cycle, we re-administered doxycycline to *MTB/TAN* or *MTB/TWNT* mice bearing residual lesions and labeled animals with BrdU. Oncogene reactivation for 48 hours resulted in high rates of tumor cell proliferation in residual lesions, demonstrating that residual tumor cells are reversibly growth-arrested and competent to re-enter the cell cycle. These data strongly suggest that residual

tumor cells in these models exist in a state of cellular dormancy. This provides a valuable *in vivo* model for tumor dormancy and provides a preclinical platform for testing our hypothesis that agents that target survival mechanisms of dormant tumor cells will result in a reduction in both minimal residual disease burden and risk of tumor recurrence.

3.2.4 GEM models for local recurrence provide insight into mechanisms of distant recurrence.

While local recurrence is itself a critical problem to understand, more than two-thirds of recurrent breast cancers in women occur at a distant site. A growing body of evidence suggests that the processes by which tumor cells – either local or distant – survive in a dormant state and ultimately re-establish malignant growth may be related mechanistically. Consistent with this possibility, local recurrence is strongly associated with an increased risk of distant relapse and mortality(25-28). Moreover, the timing of local and distant tumor relapse following surgery are similar(36). Using the above GEM models for tumor dormancy and local recurrence, we have identified several pathways that are required for mammary tumor recurrence in mice and that are also strongly associated with the risk of distant relapse in breast cancer patients. These include our findings that activation of the Notch(32) or c-MET(34) signaling pathways, up-regulation of Snail(20), or down-regulation of the tumor suppressor Par-4(33), each promote local recurrence in mice. Importantly, interrogation of gene expression data sets representing >4,000 primary human breast cancers revealed that elevated Notch activity, c-MET activity and Snail expression – and decreased Par-4 expression – each predict an increased risk of distant relapse in patients(20, 32-34). These and other observations demonstrate that GEM models for local recurrence are informative for mechanisms underlying distant, as well as local, relapse in patients.

3.2.5 Preclinical studies identify autophagy and mTOR as critical vulnerabilities of dormant tumor cells.

As described below, interrogation of our GEM models has led to the identification of autophagy and mTOR pathway activity as key mechanisms of dormant tumor cell survival and preclinical studies have demonstrated that targeted pharmacological inhibition of autophagy or mTOR depletes the reservoir of dormant residual tumor cells in mice and prevents tumor recurrence.

Autophagy inhibition prevents breast cancer recurrence by impairing the survival of dormant residual tumor cells. We tested the role of autophagy in dormant tumor cell survival in the above GEM models. Using *MTB/TAN* mammary tumor cells stably expressing the autophagy marker eGFP-LC3, we demonstrated that HER2 inhibition *in vitro* results in the induction of autophagy in residual tumor cells, as revealed by increased levels of endogenous LC3-II, decreased levels of p62, an increased number of double-membraned autophagosomes observed by electron microscopy, and relocalization of LC3 to autophagosomes. Importantly, fluorescence microscopy performed on mice harboring fully regressed eGFP-LC3-labeled orthotopic tumors revealed an increase in the number of autophagic eGFP-positive punctae per tumor cell compared to actively growing primary tumors. These results suggest that dormant residual tumor cells undergo autophagy *in vivo*.

To determine whether autophagy functionally contributes to mammary tumor recurrence, primary *MTB/TAN* tumor cells were generated expressing shRNAs targeting either *Atg5* or *Atg7*, each of which are required for autophagy. These cells were then injected orthotopically into the mammary glands of *nu/nu* mice on doxycycline, as were cells transduced with a vector control, to form primary tumors. Doxycycline was withdrawn to induce HER2/neu down-regulation and regression of tumors to a non-palpable state. Mice were then monitored for recurrence. This revealed that genetic inhibition of autophagy by knocking down either *Atg5* or *Atg7* dramatically impaired tumor recurrence (HR 5.35, 95% CI 1.72-16.62, $p = 0.004$; HR 7.56, 95% CI 2.33-24.58, $p < 0.001$, respectively). To extend these results, we determined the effects of *Beclin 1* loss on mammary tumor recurrence, since Beclin 1 is required for formation of the autophagosome. We crossed *Beclin 1*+/- mice to *MTB/TAN* mice to generate cohorts of *MTB/TAN;Beclin 1*+/+ and *MTB/TAN;Beclin 1*+/- mice. Primary mammary tumors were induced by activation of HER2/neu via doxycycline administration. The incidence, latency, multiplicity and growth rate of primary HER2/neu-induced tumors did not differ between *Beclin 1*+/+ and *Beclin 1*+/- mice. Tumor-bearing mice were then deinduced and mice bearing fully regressed tumors were monitored for recurrence. Deletion of one allele of *Beclin 1* markedly delayed tumor recurrence with the median latency for tumor recurrence increasing from 77 days to 131 days (HR 2.38, 95% CI 1.15-4.94, $p = 0.019$). In aggregate, these results strongly suggest that autophagy is required for tumor recurrence.

To address the cellular basis for this effect, we next asked what stage(s) of mammary tumor recurrence requires autophagy. *MTB/TAN* primary tumor cells expressing an shRNA targeting *Atg5* were labeled with an H2B-mCherry reporter. Control *MTB/TAN* primary tumor cells transduced with an empty vector were labeled with an H2B-eGFP reporter. These two isogenic fluorescent cell populations were admixed in equal parts, injected into the mammary glands of *nu/nu* mice maintained on doxycycline, and allowed to generate orthotopic primary tumors. Doxycycline was then withdrawn to initiate tumor regression and fluorescence microscopy was used to determine the ratio of mCherry-labeled *Atg5* knockdown cells to eGFP-labeled control cells in primary tumors, residual tumor lesions 14 d or 28 d following HER2/neu down-regulation, and recurrent tumors. As predicted based on the unperturbed kinetics of primary tumorigenesis in *Beclin 1+/-* mice, we observed no selection for or against cells with *Atg5* knockdown in primary tumors or residual lesions 14 d post-HER2/neu down-regulation, a time point roughly corresponding to the completion of tumor regression and onset of tumor dormancy. In contrast, *Atg5* knockdown cells were markedly selected against in residual lesions 28 d following HER2/neu down-regulation, a time point at which residual tumor cells have been in a dormant state for ~2 wk. This competitive disadvantage was even more pronounced in recurrent tumors, in which only 1% of tumor cells were mCherry-positive. Together, these data indicate that residual tumor cells in which autophagy is impaired are at a strong, cell-intrinsic selective disadvantage once they enter the dormant state.

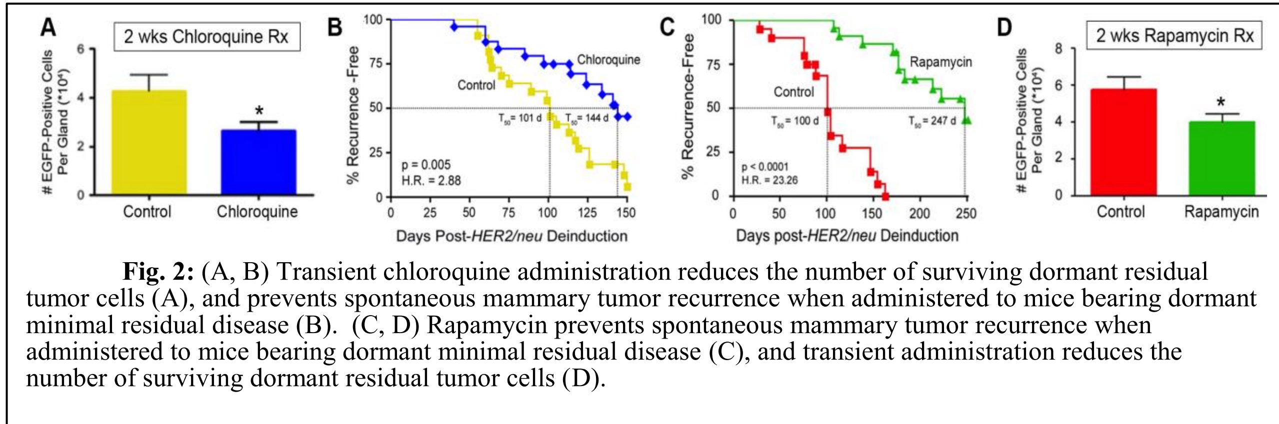


Fig. 2: (A, B) Transient chloroquine administration reduces the number of surviving dormant residual tumor cells (A), and prevents spontaneous mammary tumor recurrence when administered to mice bearing dormant minimal residual disease (B). (C, D) Rapamycin prevents spontaneous mammary tumor recurrence when administered to mice bearing dormant minimal residual disease (C), and transient administration reduces the number of surviving dormant residual tumor cells (D).

Our findings to this point suggested that genetic inhibition of autophagy impairs dormant tumor cell survival. To determine whether this was true for pharmacological inhibition of autophagy, we tested whether the anti-malarial drug chloroquine (CQ) – a known autophagy inhibitor – would phenocopy these effects and demonstrate activity against dormant tumor cells. Consistent with this possibility, dormant *MTB/TAN* tumor cells maintained in the absence of HER2/neu expression in vitro were markedly more sensitive to chloroquine treatment than proliferating cells, suggesting that dormant tumor cells are preferentially dependent upon autophagy for survival. We next tested whether dormant mammary tumors cells *in vivo* are dependent upon autophagy for survival. Mice harboring orthotopic eGFP-labeled dormant residual *MTB/TAN* tumor cells were treated with CQ for 2 wks beginning 21 days after HER2/neu down-regulation, at which time tumor cells are dormant. Mammary glands were then harvested, digested to form a single-cell suspension, and the number of eGFP-positive residual tumor cells was determined by flow cytometry. This analysis revealed that mice treated with CQ for 2 wks within the period of dormancy harbored ~40% fewer dormant tumor cells ($p < 0.05$) than mice treated with a vehicle control (Fig. 2A). Given the absence of tumor cell proliferation at these time points, these data strongly suggest that autophagy is required for the survival of dormant mammary tumor cells *in vivo*.

Our findings to this point suggested that dormant tumor cells might be uniquely dependent upon autophagy for their survival. If correct, our data would predict that inhibitors of autophagy would be more effective against dormant residual tumor cells than actively growing tumors. If so, CQ could potentially be used to reduce the pool of dormant residual tumor cells and thereby prevent or delay tumor recurrence. To test this hypothesis, CQ treatment was initiated in mice bearing dormant residual disease 28 d following HER2/neu down-regulation in orthotopic tumors. Mice were then monitored for tumor recurrence. This revealed that autophagy inhibition <u>restricted specifically to the period of tumor dormancy</u> markedly delayed tumor recurrence (H.R. = 2.88, 95% CI 1.39-5.98, $p = 0.005$) (Fig. 2B). Notably, the magnitude of the effect of CQ administration beginning 28 d after HER2/neu down-regulation was nearly identical to that observed for CQ administration beginning immediately at the time of HER2/neu down-regulation. This finding was consistent with results from the fluorescent cell competition assay indicating that cells with impaired autophagy are not selected against during primary tumor formation or within the first 14 d following HER2/neu down-regulation, during which time the vast majority of tumor cells are eliminated. This

finding was also consistent with our observations that CQ did not affect the rate of growth of primary or recurrent tumors. Together, these observations provide further support for a model in which autophagy is required for mammary tumor recurrence and in which the effects of autophagy inhibition on tumor cell survival are largely confined to the dormant state.

In summary, using genetic and pharmacological approaches, we have demonstrated that inhibition of autophagy by CQ administration, *Atg5* or *Atg7* knockdown, or deletion of one allele of *Beclin 1*, is sufficient to delay or prevent spontaneous tumor recurrence in mice bearing dormant minimal residual disease. The requirement for autophagy during tumor recurrence is attributable to its ability to serve as a survival mechanism for dormant residual tumor cells. Notably, we found that dormant mammary tumor cells are uniquely reliant upon autophagy for their survival compared to actively growing primary or recurrent tumor cells. Consistent with this cellular mechanism of action, pharmacological inhibition of autophagy in mice bearing dormant minimal residual disease significantly decreased the number of surviving dormant tumor cells and substantially delayed mammary tumor recurrence. As CQ and hydroxychloroquine (HCQ) have been used safely in millions of people worldwide for the prevention and treatment of malaria and have a favorable therapeutic index, these drugs represent an attractive approach to inhibiting autophagy *in vivo*.

3.2.6 mTOR inhibition prevents breast cancer recurrence by impairing the survival of dormant residual tumor cells.

Based on our findings that autophagy inhibition delays mammary tumor recurrence, coupled with the fact that rapamycin induces autophagy by inhibiting the mTORC1 complex, we hypothesized that rapamycin treatment would increase the number of dormant mammary tumor cells surviving HER2/neu down-regulation and thereby accelerate tumor recurrence. To determine the effect of rapamycin treatment on recurrence, eGFP-labeled *MTB/TAN* primary tumor cells were injected orthotopically into *nu/nu* mice maintained on doxycycline. Once primary tumors formed, HER2/neu was down-regulated to induce tumor regression. Treatment with 4 mg/kg rapamycin or vehicle control began at 28 d post-deinduction, when primary tumors had regressed to a non-palpable state and a small population of dormant residual tumor cells remains. Surprisingly, treatment with rapamycin dramatically delayed the latency of tumor recurrence compared to treatment with a vehicle control (HR 23.26, 95% CI 8.06-67.19, $p < 0.0001$) (Fig. 2C).

Although the finding that rapamycin delays recurrence was unexpected, given the magnitude of this effect we sought to determine its cellular mechanism. To address the possibility that rapamycin delayed the time to detection of recurrent tumors by decreasing their growth rate, rapamycin or vehicle was administered to mice harboring orthotopic *MTB/TAN* recurrent tumors. The mean growth rate of recurrent tumors was not affected by treatment with rapamycin at any time point examined, suggesting that actively growing *MTB/TAN* recurrent tumors are not affected by rapamycin treatment.

Therefore, we next asked whether rapamycin delayed mammary tumor recurrence by decreasing the survival of dormant tumor cells in vivo. To do so, we treated mice bearing dormant residual disease with rapamycin or vehicle control for 2 wk. Mammary glands from these mice were then harvested, digested to form a single

cell suspension, and the number of eGFP-labeled dormant tumor cells was determined using flow cytometry. After 2 wk of treatment, rapamycin resulted in a 30% decrease in the number of dormant residual cells present compared to vehicle control (Fig. 2D). Since dormant *MTB/TAN* tumor cells exist in the absence of proliferation, the decrease in dormant tumor cell number observed following rapamycin treatment suggests that mTOR activity is required for the survival of dormant tumor cells.

We next wished to determine if transient rapamycin treatment would exert persistent effects, as would be predicted if it reduces the number of dormant residual tumor cells. Therefore, we treated mice that harbored dormant residual disease with rapamycin for 2 wk. Treatment was then stopped and the formation of recurrent tumors was monitored. As observed with chronic rapamycin treatment, treatment with rapamycin for 2 wk significantly delayed tumor recurrence (H.R. = 2.29, 95% CI 1.27-5.17, p = 0.0088). This was consistent with our findings above that rapamycin treatment decreases the survival of dormant tumor cells. Together, these results strongly suggest that mTOR pathway inhibition by rapamycin potently prevents mammary tumor recurrence by impairing the survival of dormant residual mammary tumor cells.

3.3 Summary: Targeting dormancy with mTOR and autophagy inhibition.

Most anti-neoplastic therapeutic modalities preferentially target actively proliferating cancer cells. In light of our identification of cellular dormancy as a stage of tumor progression that is particularly reliant upon both autophagy and mTOR pathway activity for survival, our findings suggest that dormant residual tumor cells may be uniquely susceptible to agents such as HCQ and rapamycin analogues that inhibit these pathways. To date however, pre-clinical and clinical trials have principally, if not exclusively, employed autophagy inhibitors and mTOR inhibitors to target actively proliferating stages of disease, which our findings predict may be intrinsically resistant to autophagy inhibition. Accordingly, our results suggest that autophagy and/or mTOR inhibition in clinical contexts characterized by tumor dormancy may be more effective, and support autophagy and mTOR inhibition as tractable therapeutic targets for preventing breast cancer recurrence by depleting the reservoir of residual tumor cells.

3.3.1 Detection of minimal residual disease in bone marrow.

IHC analysis for CK+ cells in the bone marrow is currently the “gold standard” for identifying patients with DTCs following therapy and this assay is clinically validated insofar as it is strongly associated with risk of relapse. However, there are at least four major shortcomings of this assay: 1) it is likely to be relatively insensitive due to cell loss during the cell isolation phase of the assay; 2) it is likely to be relatively insensitive due to the inherently lower sensitivity of IHC compared to flow cytometry; 3) only CK+ cells are assayed for, neglecting the possibility that some DTCs may not express classical epithelial markers, or may express them at low levels below the limit of detection for IHC; and 4) there is limited ability to molecularly interrogate isolated cell populations of interest. Since bone marrow DTCs are strongly associated with risk of recurrent disease, and little is known about the phenotype of bone marrow DTCs in breast cancer patients, we have pursued proof-of-principle approaches in mice and human bone marrow samples to optimize approaches for detecting and isolating small numbers of DTCs with the ultimate goal of more sensitive and specific detection, improved

quantitative enumeration and the ability to interrogate DTC phenotypic and genetic properties.

To accomplish this, we have used a new in-line magnetic depletion and acoustic focusing system, the BD Biosciences FACSFocus that, combined with the BD Influx cell sorter, allows a streamlined workflow for detection and isolation of rare events to high purity. The Abramson Cancer Center Flow Cytometry and Cell Sorting Shared Resource has worked closely with BD Biosciences in the development of this system, which is currently available exclusively at Penn. The system consists of a magnetic cell separation whereby cells stained with CD45 BD IMag™ reagent are passed by a magnet placed in line with the sample injection tubing on the Influx, resulting in depletion of unwanted CD45+ cells from the marrow sample. Upon leaving the magnet, the sample then flows to a microfluidic chip with inlets for sample and wash buffer, and outlets for waste and sample. Vibrating at ~2 MHz, the chip acts as an acoustic focuser, simultaneously washing and concentrating the sample about 4-5 fold. After leaving the chip, the sample flows to the nozzle of the Influx, with electronics optimized for cell recovery. The system permits streamlined sample preparation, with minimal cell loss, and no centrifugation if directly conjugated antibodies are used. Target cell recovery is on the order of 60-80%. To test this approach in our GEM models, we crossed *MTB/TAN* mice with *TetO-TurboCre (TTC);Rosa26-lox-stop-lox-YFP (rYFP)* mice to generate *MTB/TAN;TTC;rYFP* mice in which doxycycline administration results in inducible expression of both HER2/neu and the cre recombinase in mammary epithelial cells. Consequently, doxycycline treated mice harbor fluorescently-labeled HER2/neu-induced mammary adenocarcinomas due to cre-mediated excision of the stop cassette preceding rYFP. Fluorescent primary tumors from doxycycline-induced *MTB/TAN;TTC;rYFP* mice were enzymatically dissociated and injected orthotopically into *nu/nu* mice on doxycycline to permit primary tumor outgrowth. Bone marrow cell suspensions were prepared from tumor-bearing mice by flushing the marrow cavity of tibiae and femurs, while single cell suspensions were prepared from primary tumors, lung and other tissues by enzymatic digestion and mechanical dissociation. Cell suspensions were stained with an APC-conjugated anti-CD45 antibody, followed by incubation with anti-APC magnetic particles (BD IMag). DAPI was used to identify live/dead cell populations. Primary tumor and DTC populations were isolated by FACS using the BD Influx cell sorter, in combination with novel in-line pre-enrichment technology (BD FACSFocus).

Viable DTCs were identified as YFP+/CD45-/DAPI-. Primary tumor cells and $CD45^+$ hematopoietic cells were isolated as controls. RNA was isolated, whole transcriptome amplification (WTA) performed, and expression of selected genes was examined by qPCR using TaqMan Gene Expression Assays. This analysis revealed that, as expected, DTCs isolated from mouse bone marrow or lung, as well as primary tumor cells, express YFP but are negative for CD45 expression (Fig. 3A). Conversely, isolated CD45+ cells were positive for CD45 expression, but negative for YFP expression (Fig. 3B). These results confirm our ability to identify and isolate small numbers of DTCs in bone marrow and tissues of mice. Additional experiments have confirmed our ability to perform WTA and whole genome amplification on as few as 10 cells, as well as perform expression profiling and genomic testing for 47 oncogenic

mutations occurring in human cancers.

To pilot this approach in patient samples, we spiked MCF-7 breast cancer cells into normal human bone marrow, performed RBC lysis, CD45 magnetic depletion, staining for EpCAM and HER2, and analyzed on the FACSFocus sorter. This revealed the ability to detect spiked human breast cancer cells as EpCAM+/HER2+ and CD45– (Fig. 3C).

3.4 Hydroxychloroquine (HCQ) and cancer therapy

Chloroquine (CQ) is a synthetic 4-aminoquinoline that has been used for 60 years in humans for malaria prophylaxis and treatment(58), rheumatoid arthritis(59), and human immunodeficiency virus (HIV)(60). It is an inexpensive orally available drug that has CNS penetration. It has a large therapeutic index, and its most predictable cumulative toxicity is retinopathy, which can be prevented by discontinuation of the drug(61). It is this toxicity and worldwide malarial resistance to CQ that lead to discontinuation of extensive research into CQ's non-malarial applications. Chloroquine derivatives such as HCQ are still used extensively in rheumatoid arthritis and lupus erythematosis and have a larger therapeutic index. The chemical structure of CQ derivatives allows them to serve as a weak base which is trapped in acidic cellular compartments(62). Thus chloroquine deacidifies lysosomes, inhibiting the last step in autophagy. With this last step blocked, a cell reliant on autophagy will increase the generation of autophagosomes and will eventually undergo either apoptotic or non-apoptotic cell death. Evidence in mouse models and human cancer cell lines suggest CQ may have significant anti-tumor activity by inhibiting autophagy induced by cancer therapy(46).

Adding chloroquine to improve the efficacy of anticancer therapy has already been tested in a randomized clinical trial. A small single institution placebo controlled phase III trial testing the addition of CQ at an oral daily dose of 150 mg to RT and carmustine in patients with newly diagnosed glioblastoma multiforme (GBM) yielded surprising results(49). Median overall survival was significantly longer in the CQ-treated patients (24 months) than in controls (11 months). At the end of the observation period, six patients (40%) treated with CQ were alive at 59, 45, 30, 20 (1 each) and 27 (2 patients) months after surgery. In contrast, patients in the control group survived 32, 25, and 22 months. Although not statistically significantly different, the rate of death over time was almost half as large in the CQ group compared to the placebo group (hazard ratio, 0.52 [95% CI, 0.21 to 1.26]; p= 0.139).

Hydroxychloroquine (HCQ) is commonly prescribed for rheumatoid arthritis and lupus at a dose of 400 mg po daily. A pharmacokinetic/pharmacodynamic study of escalating doses of HCQ at 400 mg/800 mg/1200 mg po daily in patients with rheumatoid arthritis followed by maintenance doses of 400 mg po daily found that doses of up to 1200 mg po daily were well tolerated(63). Dose limiting toxicities of nausea, vomiting and abdominal pain were observed at 800 and 1200 mg po daily. This toxicity correlated with blood HCQ levels, but not with blood levels of the other active metabolites, desethylhydroxychloroquine (DHCQ), desethylchloroquine (DCQ), or bisdesethylchloroquine (BDCQ). Improvement of symptoms in rheumatoid arthritis correlated with blood DHCQ levels, suggesting a dose-response relationship. Chloroquine derivatives are metabolized through the p450 enzyme system and CQ may

inhibit the metabolism of CYP2D6-metabolized drugs(64, 65).

A predictable cumulative toxicity associated with CQ is retinopathy, and this is another reason why dose escalation with CQ would be limited. While a link between HCQ and retinopathy has also been made, it occurs infrequently and only after a prolonged exposure. A study using multifocal electroretinography to detect early pre-clinical retinal changes in long-term HCQ users found that 10 out of 11 patients that developed early pre-clinical changes had been taking HCQ at doses of 400 mg po daily for greater than 5 years.(66) No overt retinopathy was noted in the 19 patients followed. This suggests that at a cumulative dose of 730 g, the risk of retinal changes increases, but techniques such as multifocal electroretinography can detect early changes and prevent overt visual loss.

3.4.1 Trials of autophagy inhibition in cancer

Autophagy is an intracellular process characterized by the formation of autophagic vesicles (AV) that sequester cytoplasmic contents and target them for degradation in lysosomes. This process is activated by metabolic stress and also by most cancer therapies (39, 45). Autophagy is used by cancer cells to remove damaged organelles and recycle macromolecules that serve as an internal reservoir of fuel in the face of a crisis of nutrient availability. Inhibition of mTOR is one of the most potent inducers of autophagy. Therapeutic activation of autophagy may be a key resistance mechanism to mTOR inhibition with Everolimus (EVE, RAD001). Dr. Amaravadi has reported *in vivo* studies that demonstrate that inhibition of therapy-induced autophagy with chloroquine derivatives enhances cell death and tumor regression (46)(5). While these studies combined chemotherapy with hydroxychloroquine (HCQ), Dr. Amaravadi has unpublished data that rapamycin combined with HCQ results in enhanced cell death compared to rapamycin or HCQ alone. Dr. Eileen White at UMDNJ has compelling evidence of synergistic cell death when CCI-779 is combined with HCQ in renal cell carcinoma cell lines and in an orthotopic mouse model of renal cell carcinoma(39) (unpublished data presented at CTEP early drug discovery meeting 2010). A number of other investigators have found that cell death induced by therapeutic interruption of growth factor cell signaling is enhanced by combination with HCQ (47, 48)(6-9). There are now 14 clinical trials registered with the NCI combining HCQ with approved or investigational anticancer agents. Investigators from multiple disciplines within cancer therapy research have become convinced that autophagy is the next important target in cancer therapy (39), and this has lead to the formation of an Autophagy in Clinical Cancer Consortium which will be conducting some of the correlative studies that are common to many of the HCQ trials.

There are 5 phase I/II clinical trials through the University of Pennsylvania involving HCQ. The most mature trial is a 15 center phase I/II trial of temozolomide/radiation/and HCQ in patients with newly diagnosed (adjuvant) GBM. This first HCQ trial was launched in 2007 after a publication of a randomized phase III trial that demonstrated a trend towards doubling of overall survival in GBM patients treated with radiation chemotherapy and chloroquine compared to patients treated with radiation and chemotherapy alone in the adjuvant setting(49). We have reported the results of our phase I/II trial in GBM at ASCO 2010 annual meeting. We found an MTD for HCQ in this combination, and also found a PK-PD interaction that demonstrated

effective HCQ-dose-dependent autophagy inhibition in patients (50). The phase II portion of the trial has completed accrual of 76 patients in 4 months.

In addition to this trial there is a phase I trial of temsirolimus and HCQ in patients with advanced solid tumors. This trial is partially sponsored by Wyeth/Pfizer. Although preliminary data cannot be included here, they are encouraging and support the preclinical findings.

3.5 Everolimus (EVE, RAD001)

Everolimus is a novel oral derivative of rapamycin. Everolimus has been in clinical development since 1996 as an immunosuppressant in solid organ transplantation and has obtained marketing authorization (Certican®) for prophylaxis of rejection in renal and cardiac transplantation in a number of countries, including the majority of the European Union. Everolimus has been in development for patients with various malignancies since 2002. Everolimus is being investigated as an anticancer agent based on its potential to inhibit tumor cell growth, proliferation, and angiogenesis. At weekly and daily schedules and at various doses explored, RAD0001 is generally well tolerated. The most frequent adverse events (rash, mucositis, fatigue and headache) associated with Everolimus therapy are manageable. Non-infectious pneumonitis has been reported with mTOR inhibitors but is commonly low-grade and reversible.

3.5.1 Everolimus mechanism of action: mTOR inhibition

Everolimus selectively inhibits mTOR a highly conserved serine-threonine kinase, which is present in all cells and is a central regulator of protein synthesis and ultimately cell growth, cell proliferation, angiogenesis and cell survival. TOR is the only currently known target of everolimus (51).TOR is downstream of PI3K/AKT pathway, a pathway known to be dysregulated in a wide spectrum of human cancers. In addition, activation of the PI3K/AKT/mTOR pathway is frequently a characteristic of worsening prognosis through increased aggressiveness, resistance to treatment and progression.

The main known functions of mTOR include the following (51, 52) 1) mTOR functions as a sensor of mitogens, growth factors and energy and nutrient levels, facilitating cell-cycle progression from G1 to S phase in appropriate growth conditions; 2) Through inactivating eukaryotic initiation factor 4E binding proteins (4E-BP1) and activating the 40S ribosomal S6 kinases (i.e., p70S6K1), mTOR regulates translation of important massages, including those encoding the HIF-1 proteins, c-myc, ornithine decarboxylase, and cyclin D1, as well as ribosomal proteins themselves; 3) The activation of mTOR pathway leads to the increased production of pro-angiogenic factors (i.e., VEGF) in tumors and to tumor, endothelial and smooth muscle cell growth and proliferation; 4) The regulation of mTOR signaling is complex and involves positive regulators, such as AKT that phosphorylate and inactivate negative regulators such as the Tuberous Sclerosis Complex (TSC1/TSC2).

Recent evidence indicates that mTOR is represented by two distinct multiprotein signaling complexes, mTORC1 (mTOR complex 1, rapamycin sensitive) and mTORC2 (mTOR complex 2, rapamycin insensitive)(53). mTORC1 is mainly activated via the PI3 kinase pathway through AKT (also known as PKB, protein kinase B) and the tuberous sclerosis complex (TSC1/TSC2) (52). Activated AKT phosphorylates TSC2, which lead

to the dissociation of TSC1/TSC2 complex, thus inhibiting the ability of TSC2 to act as a GTPase activating protein. This allows Rheb, a small G-protein, to remain in a GTP bound state and to activate mTORC1. AKT can also activate mTORC1 by PRAS40 phosphorylation, thereby relieving the PRAS40-mediated inhibition of mTORC1 (54). The second complex, mTORC2 (mTOR complex 2) is activated through a currently unknown mechanism, possibly by receptor tyrosine kinase (RTK) signaling. It has been suggested that mTORC2 phosphorylates and activates a different pool of AKT that is not upstream of mTORC1. PHLPP phosphatase plays a role of a negative regulator. mTORC2 is rapamycin insensitive and is required for the organization of the actin cytoskeleton.

The macrocyclic lactone rapamycin and its derivatives such as Everolimus modulates mTORC1-mediated signaling by binding to the 12 kDa cytosolic FK506-binding protein immunophilin FKBP12, the resulting rapamycin-FKBP12 complexes bind to a specific site near the catalytic domain of mTORC1 and inhibit phosphorylation of mTOR substrates. As a consequence, downstream signaling events involved in regulation of the G1 to S-phase transition are inhibited. This mechanism is thought to be responsible for the immunosuppressive effects of rapamycin as well as its putative antineoplastic activity (55). As many cancers are characterized by dysregulation of G1 transit (for example, overexpression of cyclin or cyclin-dependent kinases), inhibition of mTOR becomes an intriguing target for cancer therapy (52).

3.5.2 Everolimus Pharmacokinetics (PK)

Everolimus is rapidly absorbed with a median t_{max} of 1-2 hours. The bioavailability of the drug is believed to be 11% or greater. The AUC_{0-T} is dose-proportional over the dose range between 5 and 70 mg in the weekly regimen and 5 and 10 mg in the daily regimen. C_{max} is dose-proportional between 5 and 10 mg for both the weekly and daily regimens. At doses of 20 mg/week and higher, the increase in C_{max} is less than dose-proportional. The coefficient of variation between patients is approximately 50%. Trough levels (24 hour post-dose) correlate well with AUC_{0-T} at steady-state during daily administration. In whole blood, at a daily dose of 10 mg, about 20% of Everolimus is confined in plasma with 26% being unbound. The remaining 80% is sequestered in blood cells.

Everolimus is extensively metabolized in the liver and eliminated in the bile. Major metabolites are inactive. Elimination half-life is approximately 30 hours. The clearance of Everolimus is approximately halved in patients with mild-moderate hepatic impairment (Child-Pugh Class A or B), while renal impairment has little or no impact on the pharmacokinetics of Everolimus.

Age, weight and gender in the adult population do not affect the pharmacokinetics of Everolimus to a clinically relevant extent. The clearance of Everolimus is reduced in children. Pharmacokinetic characteristics are not notably different between Caucasian and Japanese subjects, whereas in Black patients, population pharmacokinetic studies have shown an average 20% higher clearance.

A high-fat meal altered the absorption of Everolimus with 1.3 hour delay in t_{max}, a 60% reduction in C_{max} and a 16% reduction in AUC. Everolimus is a substrate of CYP3A4 and a substrate and a moderate inhibitor of the multi-drug efflux pump P-

glycoprotein (P-gP, MDR1, ABCB1). Hence, its metabolism is sensitive to drugs that modify these enzymes (substrates, inducers, or inhibitors of these enzymes). Competitive inhibition could occur when Everolimus is combined with drugs which are also CYP3A4 or P-glycoprotein substrates. Table 10 (Section 7.3) lists examples of clinically relevant CYP3A inhibitors and inducers. Please refer to Section 7.3 for more information on the concomitant use of CYP3A4 inhibitors/inducers and other medications. More information on Everolimus pharmacokinetics is provided in the [Investigator's Brochure].

3.5.3 Everolimus pharmacodynamic studies

PK/pharmacodynamic modeling based on inhibition of the biomarker p70S6 kinase 1 [S6K1] in peripheral blood mononuclear cells [PBMC] suggests that 5-10 mg daily should be an adequate dose to produce a high-degree of sustained target inhibition ([Study C2101] / [Study 2102], Lane, et al 2003). Furthermore, molecular pharmacodynamic (MPD) studies, using immunocytochemistry (IHC) in biopsied tumor tissue, assessed the degree of inhibition and its duration for pS6, p4E-BP1 and pAKT expression with the daily and weekly dosing. There was high inhibition of the downstream markers S6K1 and 4E-BP1 at 5 mg/day, which was complete at 10 mg/day, while preliminary results suggest increase in pAKT expression with maximal effect at 10 mg daily ([Study C2107]). More information is provided in the [Investigator's Brochure].

3.5.4 Clinical trials of Everolimus in cancer patients

Everolimus has been in development for patients with cancer since 2002. Everolimus (Afinitor®) was approved by the United States Food and Drug Administration in March 2009 for the treatment of advanced renal cell carcinoma (RCC) after failure of treatment with sunitinib or sorafenib. In 2010, Afinitor® was received United States (US) approval for the treatment of patients with subependymal giant cell astrocytoma (SEGA) associated with tuberous sclerosis complex (TSC). Everolimus is also available as Votubia® in the European Union (EU) for patients with SEGA associated with TSC who require therapeutic intervention but are not candidates for curative surgical resection. Afinitor® was approved for "progressive pancreatic neuroendocrine tumor (PNET) in patients with unresectable, locally advanced, or metastatic disease" in 2011 in various countries, including the US and Europe. In 2012 Afinitor® received approval for the treatment of postmenopausal women with advanced hormone receptor-positive, HER2- negative breast cancer (advanced HR+ BC) in combination with exemestane, after failure of treatment with letrozole or anastrozole. Furthermore in 2012, Afinitor® received approval for the treatment of patients with TSC who have renal angiomyolipoma not requiring immediate surgery. In the US, Afinitor® was approved in 2016 for advanced non-functional NET of gastrointestinal (GI) or lung origin.

Approximately 112,317 cancer patients have been enrolled in studies with everolimus as of 31-Mar-2017(this number excludes patients who received marketed Afinitor®/Votubia®/Afinitor DISPERZ, those on planned and roll over studies as well as those on investigator-sponsored studies:

- 110,217 patients in Novartis-sponsored clinical trials

- 2,100 patients in the individual patient supply program

Approximately 47,349 patients were enrolled globally in all investigator-sponsored studies as of 31-Mar-2017.

Eight single-agent Novartis sponsored trials have or are being conducted in various advanced malignancies. Five Phase I studies evaluated several escalating doses with either weekly or daily administration (Studies C2101/02, C2106, C2107, C1101) of Everolimus determined that the 10 mg/day and 50-70 mg/week dosages were proposed for further studies, when using Everolimus as a single agent, and as a target maximum dose in combination studies. Two Phase II monotherapy studies were designed to evaluate the safety and efficacy of a single dose of 10 mg administered daily including Study C2235 in advanced NSCLC (n=81) and Study C2239 in advanced pancreatic neuroendocrine tumors (n=160).

A Phase III, randomized, double blind, placebo controlled study in patients with mRCC who progressed on a VEGFr TKI demonstrated that everolimus 10 mg daily (Motzer, 2008) prolonged median PFS from 1.87 months for patients receiving placebo to 4.01 months for everolimus-treated patients, assessed by central independent review blinded to clinical data (hazard ratio 0.30, 95% CI 0.22-0.40, $p<0{\cdot}0001$)(56). Updated results presented at the American Society of Clinical Oncology 2009 Genitourinary Cancers Symposium (Kay et al, 2009) demonstrated everolimus as having even greater superiority to placebo in the primary endpoint of PFS. Median PFS was prolonged from 1.9 months for patients receiving placebo to 4.9 months for everolimus-treated patients, assessed by central independent review blinded to clinical data (hazard ratio 0.33, 95% CI 0.25-0.43,

Table 1: Adverse Events for Combination HCQ/EVE in Advanced Renal Cell Carcinoma

p<0.001)(57).

Overall, the most frequent adverse effects suspected to be related to Everolimus have been stomatitis, rash, anemia, fatigue, asthenia, diarrhea, anorexia, nausea, hypercholesterolemia, mucosal inflammation, vomiting, hypertriglyceridemia, cough, peripheral edema, dry skin, epistaxis, pruritus and dyspnea. The most common Grade 3 or 4 adverse reactions suspected to be related to treatment were anemia, infections, hyperglycemia, stomatitis, fatigue, lymphopenia, hypercholesterolemia, pneumonitis, and elevated gamma-glutamyl transferase concentrations.

Non-infectious low-Grade (Grade 1/2) pneumonitis has led to development of treatment guidelines for the disorder (Table 4). The primary DLT has been severe (Grade 3) stomatitis, and occasionally fatigue, hyperglycemia, and neutropenia. For more information on known undesirable effects of Everolimus refer to Section 7.2.

Further detailed information regarding Everolimus clinical development, safety and efficacy is provided in the [Investigator's Brochure].

Adverse events for a phase I/II trial of Evererlimus and HCQ in advanced renal cell carcinoma (N=34)				
	Grade 1-2		Grade 3-4	
Adverse event	N	%	N	%
Anemia	11	32	0	0
Anorexia	8	24	1	3
Blurred vision	1	3	0	0
Constipation	3	9	0	0
Diarrhea	7	21	0	0
Dysgeusia	2	6	0	0
Edema	3	9	2	6
Elevated creatinine	7	21	0	0
Fatigue	10	29	0	0
Headache	4	12	0	0
Hypercholesterolemia	3	9	0	0
Hyperglycemia	2	6	2	6
Hypertriglyceridemia	6	18	2	6
Hyponatremia	2	6	0	0
Insomnia	4	12	0	0
Mucositis	5	15	0	0
Nausea	13	38	0	0
Rash	8	24	0	0
Thrombocytopenia	5	15	0	0
Vomiting	4	12	0	0

3.6 Combination therapy with HCQ and EVE

Translation from mouse to human studies required selection of agents for inhibition of autophagy and mTOR that are safe and specific. Though chloroquine was used in our mouse preclinical studies, in humans it produces sudden death in infants and severe retinopathy. Hydroxychloroquine (HCQ) is a safe alternative that is an established anti-malarial and anti-inflammatory used in rheumatoid arthritis and lupus erythematosis. It is orally administered and has rapid gastrointestinal absorption and renal excretion. Dr. Ravi Amaravadi, (co-investigator on this proposal), has demonstrated autophagy inhibition by HCQ by EM and several studies by Amaravadi and others have shown that HCQ augments the cytotoxicity of a number of chemotherapies and targeted therapies[54]. Thus, HCQ would be expected to inhibit autophagy, thereby killing BM-DTCs that are relying on autophagy for survival. Temsirolimus (TEM) and Everolimus (EVE) each inhibit mTOR through allosteric binding to mTORC1[55]. Further, mTORC1-ULK1 complex interactions can directly upregulate autophagy, an effect that is blocked by suppression of mTORC1 activity[56], thus potentially enhancing HCQ effects on

autophagy inhibition. We collaborated with Dr. Amaravadi on a phase I trial of the TEM/HCQ combination in advanced solid tumors, which also demonstrated autophagy inhibition[57]. But while TEM/HCQ was tolerable, EVE has the advantage of an all-oral regimen, and is already FDA approved in the metastatic setting[58,59]. Dr. Amaravadi has now completed accrual to a phase I/II trial in advanced renal cell carcinoma (NCT01510119), demonstrating the safety and feasibility of the HCQ/EVE combination Three patients received HCQ at 400 mg twice daily, followed by an additional 31 patients who received the target HCQ dose of 600 mg twice daily and concurrent EVE at 10 mg daily. The combination was well tolerated. The only grade 3/4 AEs were anorexia (1 patient), and edema, hyperglycemia and hypertriglyceridemia (each in 2 patients).

3.7 Rationale for the proposed proof-of-concept pilot trial

The data presented above provide compelling evidence that the source of relapse in some breast cancer patients may be cells that have entered a state of dormancy, and exist in a state distinct from the primary tumor from which they originated, both biologically and with respect to their response to therapies. Currently, there are no adjuvant therapies that specifically target dormancy or take advantage of the unique vulnerabilities of minimal residual disease, despite the fact that the vast majority of breast cancer patients reside in this state. The proposed investigations take our novel preclinical findings to the critical next stage: redefining the paradigm of adjuvant therapy by targeting residual dormant disease with agents specifically identified to block key survival mechanisms in these cells, mTOR and autophagy.

Our preclinical studies in mice showing prevention or delay of recurrence with CQ and rapamycin provide a convincing rationale for translating these findings to the clinic. Furthermore, the existence of a clinically validated IHC assay for DTCs to identify at-risk patients, our initial work to develop an improved DTC assay, and phase I/II data demonstrating the safety and feasibility of treating cancer patients with HCQ and EVE, have together laid the groundwork needed to conduct this novel, innovative clinical trial. Our multidisciplinary team will work closely to translate findings bi-directionally from concurrent human and mouse studies and, in doing so, will further understanding of the biology of dormancy, develop improved methods for identifying and analyzing human DTCs, and operationalize our platform to bring the next generation of clinical trials targeting dormancy to the clinic. This approach has the potential to provide the "breakthrough" needed to eradicate recurrent breast cancer and provide the tools and evidence needed to give every breast cancer patient the ability to be monitored actively and to take proactive measures for secondary prevention.

3.8 Trial Overview

To translate findings from our mouse preclinical studies to humans, we developed a regimen targeting autophagy and mTOR signaling that is safe, effective and feasible in a potentially curative setting. HCQ is an established anti-malarial that is also used in chronic inflammatory diseases such as rheumatoid arthritis, lupus erythematosis and others. It is orally administered and has rapid gastrointestinal absorption and elimination by the kidney. Autophagy inhibition with HCQ has been shown to augment the cytotoxicity of a number of chemotherapies and targeted

therapies(39). Temsirolimus (TEM) and Everolimus (EVE) are rapamycin derivatives that inhibit mTOR through allosteric binding to mTORC1(40). Further, mTORC1-ULK1 complex interactions can directly regulate autophagy, an effect that is blocked through the suppression of mTORC1 activity(41), thus potentially enhancing the effect of HCQ in inhibiting autophagy. To generate initial safety data and assess target inhibition, we performed a phase I trial of TEM and HCQ in advanced solid tumors(42). Patients received oral HCQ at 200 – 1200 mg daily in divided doses and temsirolimus, 25 mg intravenously on a weekly schedule. In the 1200 mg HCQ cohort (n=16), the most common grade 3/4 events were nausea (n=4), anorexia (n=2), and dysguesia, elevated creatinine, fatigue, anemia and lymphopenia, each in 1 patient. Other common grade 1/2 toxicities seen in the overall study cohort included constipation (13%), diarrhea (18%), rash (38%), stomatitis (41%) and weight loss (18%). Thus, this was a tolerable regimen with no severe safety signals. In addition, tissue studies demonstrated that this combination effectively inhibited target. However, moving into the surveillance setting in breast cancer, an all-oral regimen is more feasible, appealing to patients, and titratable in a potentially curative setting. Moreover, EVE has been studied extensively in breast cancer patients, demonstrating safety and activity in combination with both endocrine and HER2-directed therapies(43, 44). Thus we sought data on the safety and efficacy of HCQ with EVE. Dr. Amaravadi (sub-investigator on this proposal) has now completed dose escalation on a phase I/II study of HCQ and EVE in patients with advanced renal cell carcinoma (NCT01510119). In 29 patients, there were no SAEs or grade 4 toxicities, two grade 3 episodes each of nausea and edema, and one episode elevated triglycerides. These data confirm that HCQ at 600 mg twice daily and EVE at 10 mg daily are safe and tolerable.

4 PATIENT SELECTION

4.1 Target enrollment and subject ascertainment

A total of 60 patients in the post-treatment surveillance period with bone marrow positive for DTCs will be ascertained from the ongoing PENN-SURMOUNT Screening Study (UPCC 28115; IRB# 824098) or other studies in which they have the DTC-IHC assay performed in the Clinical Laboratory of the Department of Pathology at the University of Pennsylvania. Patients on UPCC 28115, the ongoing screening study, meet the same clinical eligibility criteria as outlined for the clinical trial below. Screening DTC results (IHC assay) will be made available to the participant’s study doctor, who will discuss them with the participant. If the screening DTC result is positive, the proposed clinical trial will be discussed.

4.2 Inclusion criteria

4.2.1 Histologically-confirmed, primary, invasive breast cancer diagnosed within 5 years of entry into a companion DTC screening protocol.

4.2.2 Qualifying risk status, at diagnosis utilizing receptor testing by ASCO/CAP guidelines, meeting one of the following:

4.2.2.1 Histologically positive axillary lymph nodes

4.2.2.2 Primary tumor with triple negative subtype: estrogen receptor (ER) <10%, progesterone receptor (PR) <10% and negative Her2-overexpression by ASCO-CAP guidelines

4.2.2.3 Primary tumor with Breast Cancer Recurrence Score of ≥ 25 per the Genomic Health Oncotype DX breast cancer test and/or high risk MammaPrint

4.2.2.4 Evidence of residual disease in the breast on pathological assessment after neoadjuvant chemotherapy.

4.2.3 Patients must have completed all primary therapy (definitive surgery, (neo)adjuvant chemotherapy adjuvant radiation and/or Her2-directed therapy) for the index malignancy at least 4 weeks prior to study entry. All prior treatment-related toxicity must be resolved prior to study enrollment. Concurrent receipt of adjuvant endocrine and bone modifying agents is allowed per standard of care guidelines. Tamoxifen is not allowed due to drug-drug interactions with HCQ (see Section 7.6 and Table 3).

4.2.4 Bone marrow aspirate after completion of therapy demonstrates detectable DTCs (via IHC)

4.2.5 No evidence of recurrent local or distant breast cancer by physical examination, blood tests (CBC, LFTs, Alk Phos), or symptom-directed imaging, per NCCN guidelines.

4.2.6 Age ≥ 18 years

4.2.7 ECOG performance status ≤ 2

4.2.8 No contraindications to the study medications or uncontrolled medical illness.

4.2.9 Adequate bone marrow function as shown by: ANC ≥ 1.5 x 10^9/L, Platelets ≥ 100 x 10^9/L, Hb >9 g/dL

4.2.10 Adequate liver function as shown by: Serum bilirubin ≤ 1.5 x ULN, ALT and AST ≤ 2.5 x ULN, and INR ≤1.5

4.2.11 Anticoagulation is allowed if target INR ≤ 1.5 on a stable dose of warfarin or on a stable dose of anticoagulant for >2 weeks at time of randomization. For patients on therapeutic anti-coagulants, medication must be clinically held peri-procedure (bone marrow aspirate) per standard clinical management.

4.2.12 Adequate renal function: serum creatinine ≤ 2.0 x ULN or creatinine clearance (CrCl) ≥ 30mL/min obtained within 28 days prior to registration. A calculated creatinine clearance by Cockcroft-Gault Formula is acceptable in lieu of a measured value.

4.2.13 Serum cholesterol ≤300 mg/dL OR ≤7.75 mmol/L AND triglycerides ≤ 2.5 x ULN. NOTE: In case one or both of these thresholds are exceeded, a fasting lipid assessment will be ordered. If one or both of these thresholds are still exceeded in a fasting state, the patient can only be included after initiation of appropriate lipid lowering medication.

4.2.14 Ability to provide informed consent

4.3 Exclusion criteria

4.3.1 Concurrent enrollment on another investigational therapy

4.3.2 Prior treatment with an mTOR inhibitor (sirolimus, temsirolimus, everolimus).

4.3.3 Known hypersensitivity to Everolimus or other rapamycins (sirolimus, temsirolimus) or to its excipients.

4.3.4 Known allergies or hypersensitivity to corticosteroids

4.3.5 Patients receiving chronic, high dose systemic treatment with corticosteroids defined as: chronic use of cortisone >50mg; hydrocortisone >40mg, prednisone >10mg, methylprednisone >8mg or dexamethasone > 1.5mg; or another immunosuppressive agent. Topical or inhaled corticosteroids are allowed.

4.3.6 Patients should not receive immunization with attenuated live vaccines within one week of study entry or during study period.

4.3.7 Patients who have any severe and/or uncontrolled medical conditions or other conditions that could affect their participation in the study including:

- Symptomatic congestive heart failure of New York heart Association Class III or IV
- Unstable angina pectoris, myocardial infarction within 6 months of start of study drug, serious uncontrolled cardiac arrhythmia or any other clinically significant cardiac disease
- Severely impaired lung function with a previously documented spirometry and DLCO that is 50% of the normal predicted value and/or 0_2 saturation that is 88% or less at rest on room air
- Uncontrolled diabetes as defined by fasting serum glucose >1.5 x ULN. For eligibility assessment, if non-fasting serum glucose ≤ 1.5 x ULN, then a fasting serum glucose does not need to be obtained.
- Active (acute or chronic) or uncontrolled severe infections
- Liver disease such as cirrhosis, chronic active hepatitis or chronic persistent hepatitis
- A known history of HIV seropositivity as reported by the patient

- Impairment of gastrointestinal function or gastrointestinal disease that may significantly alter the absorption of EVE (e.g., ulcerative disease, uncontrolled nausea, vomiting, diarrhea, malabsorption syndrome or small bowel resection)
- Patients with an active, bleeding diathesis
- Active or latent, untreated Hepatitis B or C. A detailed assessment of Hepatitis B/C medical history and risk factors must be done at screening for all patients. HBV DNA and HCV RNA PCR testing are required at screening for all patients with a positive medical history based on risk factors and/or confirmation of prior HBV/HCV infection.

4.3.8 Female patients who are pregnant or breast feeding, or adults of reproductive potential who are not using effective birth control methods. If barrier contraceptives are being used, these must be continued throughout the trial and for 8 weeks after stopping study drug, by both sexes. Hormonal contraceptives are not acceptable as a sole method of contraception. (Women of childbearing potential must have a negative urine or serum pregnancy test within 7 days prior to administration of EVE. Pregnancy testing is required unless age ≥ 60, prior hysterectomy, prior oophorectomy, ≥ 24 months amenorrhea, or patient is between 12-24 months amenorrhea and on anti-estrogen therapy with estradiol < 30 pg/mL and FSH in post-menopausal range)

5 STUDY ASSESSMENTS AND PROCEDURES

5.1 Schedule of Assessments and Procedures

The table below lists the procedures and assessments that patients must undergo as part of this pilot clinical trial.

Table 2: Study Calendar

	Screening Phase[1]	**Treatment Phase**	**First Follow-Up**	**Follow-Up Phase**
Cycle (C) **1 cycle = 28 days**	0 (baseline)	Each Cycle	30 days after last dose IP therapy	Q6 month x 3 years
Day (D) of Cycle		D1[4]		
Time Window (days)	-30 to 0	+/- 3	+/- 15	+/- 35
SCREENING PHASE:				
Informed Consent	X			
Inclusion/Exclusion Criteria	X			
Demographics	X			
CLINICAL EVALUATIONS:				
Medical History	X			
Physical Exam/Vital Signs	X	X	X	X
ECOG Performance status	X	X	X	
LABORATORY/RADIOLOGIC EVALUATIONS:				
Hematology (CBC/diff, plt)	X	X	X	X
Comprehensive Metabolic Panel[2]	X	X	X	X
Coagulation Studies (PT/PTT)	X			
Serum Pregnancy Test[3]	X			
TREATMENT ADMINISTRATION:				
IP Dispensing, Drug Compliance		X		
CORRELATIVE STUDIES:				
Breast tumor specimen (primary tumor)	X			
Bone Marrow Aspirate for DTCs	X[7]	X[5]		X[8]
Blood sample for patient tumor DNA and CTCs	X	X[6]		X
ADDITIONAL INFORMATION:				
Concomitant Medications Review	X	X	X	X
Adverse Events/Toxicity Assessment	X	X[4]	X	

1. Within 30 days prior to starting treatment, unless otherwise noted.
2. Serum chemistry includes measurement of sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, calcium, total protein, albumin, AST, ALT, and alkaline phosphatase. In addition to the standard blood chemistry (complete metabolic panel), a lipid panel will be done to measure serum cholesterol and triglycerides. If any lipid assessment exceeds the threshold identified in eligibility criterion 4.2.13, then a fasting lipid panel will be ordered. Magnesium and phosphorous levels will be evaluated at screening for all subjects, and subsequently during the treatment phase and at first follow-up for those patients receiving everolimus.
3. For women of childbearing potential only, within 7 days of initiating treatment.
4. Participants randomized to Arm D will be monitored remotely by phone during the 12 week observation period. Lab assessments are not required during the observation period.
5. Bone marrow aspirate performed at the end observation (before the start of therapy), at the end of C3 (between D23-28), at the end of C6 (between D23-21 days after last dose IP therapy), and, if patient continued on additional treatment, at the end of C12, (between D23-21 days after last dose IP therapy).
6. Blood collected for ptDNA and CTCs pretreatment and at the completion of observation (before the start of therapy), at the completion of C3 (before the start of C4), at the completion of C6 (within 21 days of last dose), and at completion of C12 (within 21 days of last dose).
7. Baseline bone marrow aspirate positive for DTCs must have been done within 6 months of the screening phase.
8. Participants who clear DTCs from the bone marrow (i.e., negative DTC-IHC test) after 6 or 12 cycles of therapy will have optional bone marrow aspirate collected for retesting at the first 6 month follow up visit.

Note: Additional tests may be performed at the discretion of the treating investigator as clinically indicated. The sample collection schedules outlined above are based on an ideal subject. The sample schedule should be followed as closely as is realistically possible; however, the schedule may be modified due to problems such as scheduling delays or conflicts (e.g., clinic closure, poor weather conditions, vacations, etc.)

5.2 Screening Phase

Patients found to have detectable DTCs by immunohistochemistry, through the PENN-SURMOUNT Screening study (UPCC 28115; IRB# 824098) or other studies will be screened for trial participation during the screening phase. This will include assessments of medical history, overall physical condition, physical exam, baseline laboratory studies, and symptom-directed imaging (if applicable) to rule out the presence of overt metastatic disease, per NCCN guidelines. Patients will undergo a baseline bone marrow aspirate if their prior bone marrow aspirate detecting DTCs occurred > 6 months prior to the screening phase. If a bone marrow aspirate positive for DTCs occurred ≤ 6 months prior to screening, this will not be repeated, and the prior DTC count will provide the baseline DTC assessment.

5.3 Treatment Phase

Patients who have signed informed consent will be randomized to start the treatment phase. The study arms are described below. Patients are assessed for adverse events, toxicity and laboratory abnormalities at each study visit. Bone marrow aspirate will be performed at the end of the third cycle of observation, between D23-28 (Arm D), at the end of 3 cycles of therapy, between D23-D28 (all arms), after 6 cycles of therapy, between D23 of C6 and within 21 days of the last dose of C6 (all arms), and, if patient continues on treatment, at the end of C12, between D23 of C12 and within 21 days of the last dose of C12. After the first 6 cycles of study therapy, patients will stop all study medication and proceed with

bone marrow evaluation per protocol. When the results are available it will be determined whether or not the patient will continue on study therapy. If continuing on study therapy, Cycle 7 must be initiated within 45 days after the last dose of cycle 6. Results from the end of C3 and end of observation bone marrow tests will not be returned to patients. Patients, however, will receive the results of the end of C6 and, if applicable and if desired, end of C12 bone marrow tests. End of C6 and, if applicable, end of C12 bone marrow DTC results disclosure can be done remotely over the phone if deemed appropriate by the PI or treating physician.

5.4 First Follow-Up and Follow-Up Phase

Patients will be seen in follow-up 30 days after their last dose of study treatment (“first follow-up”) and then at 6 month intervals x 3 years. At these visits, symptoms related to residual side effects of study treatment or potentially reflecting recurrent disease will be queried and physical examination will be performed. AEs will be collected up until 30 days after the last dose of study treatment. Any unresolved AEs will be followed until resolved to grade ≤1 or baseline, or can be otherwise attributed. Collection of new AEs (other than those related to study procedures) beyond the first follow up visit is not required. Blood will be collected for ptDNA and CTC at 6 month intervals x 3 years. There is an optional bone marrow aspirate collection for DTC testing at the first 6 month follow up visit for patients who had a negative DTC-IHC test after 6 or 12 cycles of therapy. Patients will have the opportunity to receive the results of this bone marrow test. Patients may also choose not to receive the results of this bone marrow test, in which case the results will not be placed in the EMR.

To decrease the burden as much as possible on subjects, subjects may go to their local oncologist for all follow up visits beyond the 30 day “first follow up.” Records will be requested from local care providers so that the study team can continue to track the subjects for safety and outcome. To minimize inconvenience and burden, and to improve compliance, subjects have the option of having their clinical labs drawn locally. For those subjects who are approved for remote follow up with their local providers, the research blood requirement and any other labs that are billed to research and not considered SOC will be waived. However, all subjects will retain the option of returning to UPenn if they would like to undergo study research blood collection.

5.5 Laboratory and adverse event assessment

Patients randomized to Arms A, B or C will be evaluated at the start of each treatment cycle for both toxicity assessment and laboratory evaluation. Patients who are randomized to control (Arm D) will be contacted by phone at the start of each observation cycle as a check-in and to monitor for adverse events. Laboratory evaluation is not necessary for patients on Arm D during the observation period. When Arm D patients return to start combination treatment, they will undergo toxicity assessment and laboratory evaluation at the start of each treatment cycle.

5.6 Disease monitoring

The goal of the proposed trial is to pharmacologically target dormant tumor

cells to prevent recurrent breast cancer. To enter the trial, patients must have no evidence of recurrent local or distant breast cancer by physical examination, blood tests (CBC, LFTs, Alk Phos), or symptom-directed imaging, per NCCN guidelines. This testing is repeated 6 months after the last dose of study treatment to determine whether the patient has experienced a recurrence during the study period or follow up period. No further imaging will be done as part of study procedures during or after the trial, as detection of overt metastatic disease and time-to-event analyses are beyond the scope of this pilot trial.

6 ENROLLMENT PROCEDURES

6.1 Guidelines for Enrollment

The following are required to enroll a patient on the trial:

1) Copy of the patient's signed and dated Informed Consent including documentation of the consent process
2) Patient Enrollment Form
3) Complete provided protocol-specific eligibility checklist using the eligibility assessment documented in the participant's medical/research record. To be eligible for registration to the study, the participant must meet each inclusion and exclusion criterion listed in the eligibility checklist.
4) Tissue Block Registration

The Study Coordinator or designate will assign sequence numbers to all patients in screening. Only patients deemed eligible will be registered to investigational treatment. Sequence numbers will not be re-used if a patient screen fails. Following registration, eligible participants should begin study treatment consistent with the protocol no later than 30 days after registration by the Study Coordinator. If a participant does not receive protocol therapy following registration within the allowed time period, the participant's registration on the study may be canceled. The Project Manager should be notified of cancellations as soon as possible.

Issues that would cause treatment delays should be discussed with the Principal Investigator. If a participant does not receive protocol therapy following enrollment within allowed time period, the participant will become ineligible and will be removed from the study. Such patients will have to undergo re-screening in order to participate in the study. Any requests for eligibility exceptions and/or deviations must be approved in writing by the Principal Investigator, the Medical Monitor and the IRB.

As is generally accepted, standard of care procedures performed prior to consent, but within the protocol defined screening window for each assessment, can be used for study purposes. All research-only procedures must be performed after the consent date.

7 TREATMENT PLAN

This is a randomized, controlled, open label pilot trial. A total of 60 patients

will be enrolled, with at least 15 patients allocated to each treatment group (Arms C and D constituting the same treatment group). The initial treatment period is 6 months (24 weeks). During the initial treatment period, patients will be randomized to one of 4 arms: HCQ alone (Arm A), EVE alone (Arm B), combination HCQ and EVE (Arm C) – Arm C is closing to accrual with Amendment 7 – or control/delayed combination HCQ and EVE (Arm D). Starting with Amendment 7, patients who would have been allocated to Arm C will be redirected to Arm D in order to increase the number of patients with repeat DTC assessments to aid assay reproducibility analysis At the conclusion of 3 cycles of therapy (Arms A, B, and C) or 3 cycles of observation (Arm D), patients will undergo repeat bone marrow aspirate. Patients on Arm D will then commence combination therapy with HCQ + EVE for 6 cycles (24 weeks). After 3 cycles of therapy, patients on Arm D will undergo another bone marrow aspirate. After 6 cycles of therapy, patients on all arms will undergo another bone marrow aspirate. Patients with a negative bone marrow aspirate for DTCs at after cycle 6 will have all therapy discontinued and enter the follow up phase. Patients who have persistently positive bone marrow DTCs will be continued on combination HCQ + EVE for an additional 6 cycles (24 weeks). At the conclusion of this combination treatment period (cycle 12), all treatment will be discontinued and a bone marrow aspirate will be performed.

Patients will be evaluated at the initiation of every cycle for adverse events, and dose reductions will be employed for toxicity. Patients will continue to be followed for recurrence events for 3 years after completion of treatment.

7.1 Study Arms

Patients will be randomized during the Screening Phase to one of four investigational arms. These are shown in the schema above and detailed as follows (Note that Arm C is closing with Amendment 7):

Arm A: Patients will receive HCQ, 600 mg BID, for 24 weeks. If they are determined to have detectable DTCs after week 24, they will be crossed over to combination therapy, in which they will continue HCQ, 600 mg BID and add EVE, 10 mg daily for an additional 24 weeks.

Arm B: Patients will receive EVE, 10 mg daily, for 24 weeks. If they are determined to have detectable DTCs after week 24, they will be crossed over to combination therapy, in which they will continue EVE, 10 mg daily and add HCQ, 600 mg BID, for an additional 24 weeks.

Arm C: Patients will receive the combination of HCQ, 600 mg BID, and EVE, 10 mg daily, for 24 weeks. If they are determined to have detectable DTCs after week 24, they will continue combination HCQ/EVE therapy for an additional 24 weeks. Arm C is closing with Amendment 7. Patients radnomized after Amendment 7 who would have been assigned to Arm C will be redirected to Arm D.

Arm D: Patients will be observed for 12 weeks without study therapy. At the completion of the observation period, they will receive combination therapy of HCQ, 600 mg BID and EVE, 10 mg daily for 24 weeks. If they are determined to have detectable DTCs after week 24, they will continue combination HCQ/EVE therapy for an additional 24 weeks.

7.2 Drug Administration

Patients will be randomized to receive HCQ, EVE, the combination, or observation (control)/delayed combination. A “cycle” is 28 days (4 weeks) in duration. Both agents will be given continuously (28 of 28 days), without break, unless necessary due to toxicity. Patients will initially be treated on the assigned arm for 6 cycles (24 weeks), or observed for 3 cycles (Arm D), followed by delayed treatment on the combination. At that point, patients who are persistently positive for bone marrow DTCs will be allowed to crossover as outlined in the study schema and below. The crossover therapy will be given for 6 cycles (24 weeks) as well. Because EVE and HCQ are oral medications, pill counts and treatment diaries will be used to monitor adherence to therapy. Patients are required to bring the pill bottles and treatment diaries to each visit.

7.2.1 Hydroxychloroquine

Patients randomized to an HCQ-containing arm will receive HCQ at a dose of 600 mg orally twice daily. Capsules of HCQ are available in 200 mg strength, thus patients will initially start with 3 capsules twice daily for a total of 6 capsules per day. HCQ will be administered in divided doses (BID). When taking HCQ twice daily, the two daily doses should be taken 12 hours apart as close to 9am and 9pm as possible and documented clearly on the patient calendar. Patients receiving antacids, sucralfate, cholestyramine, and/or bicarbonate should have the HCQ drug dose administered at least 1 hour before or 2 hours after these medications. Hydroxychloroquine will be obtained through a written prescription by study MD as an initial 28- day supply and refilled every cycle. Patients will be required to bring the prescription to their visit on day 1 of each cycle, along with previous months’ pill container so that pill counts can be performed. In addition, treatment diaries will be used to monitor adherence to therapy. Patients are required to bring the pill bottles and treatment diaries to each visit. Missed or vomited doses should not be made up; patient should resume planned dose at next dosing interval.

7.2.2 Everolimus

Patients randomized to an EVE-containing arm will receive EVE as a dose of 10 mg orally daily, on a continuous basis. Patients will be instructed to take EVE in the morning, at the same time each day. EVE may be taken with or without food. If vomiting occurs, no attempt should be made to replace the vomited dose. All dosages prescribed and dispensed to the patient and all dose changes during the study must be recorded. EVE will be provided by Novartis Pharmaceuticals Corporation. Antiemetic prophylaxis is not required. However, control of nausea and vomiting may require institution of multiple anti-emetic medication including phenothiazines or 5-HT3 antagonists. Due to its interaction with P450 enzymes, aprepitant use should be avoided.

7.3 Adjuvant Endocrine Therapy

Patients with hormone receptor positive primary or residual tumor will continue on their assigned adjuvant endocrine therapy throughout the duration of the trial. Note that tamoxifen is not allowed during the study due to drug-drug interactions with HCQ (see Section 7.6 and Table 3).

7.4 Concomitant Treatment and Supportive Care Guidelines

Necessary supportive measures for optimal medical care will be provided during the study (including administration of prophylactic antiemetic medication if

considered appropriate by the Investigator).

7.4.1 Antiepileptics:

Because HCQ has known effects on P450 enzymes, patients requiring anti-convulsants may be treated with any of the non-enzyme inducing anti-convulsants which include: felbamate, valproic acid, gabapentin, lamotrigine, tiagibine, topiramate, zonisamide, or levetiracetam.

7.4.2 Hematologic Growth Factors

The use of hematologic growth factors (filgrastim, pegfilgrastim, epoetin alfa) is permitted to provide optimal care for patients with severe myelosuppression.

7.4.3 Bone targeting therapies

Bone targeting therapies such as bisphosphonates and RANK lignad inhibitors may be administered per standard of care guideliens. Zometa or denosumab may be given if clinically indicated.

7.5 Concomitant Medications

The case report form must capture the concurrent use of all other drugs, over-the-counter medications, or alternative therapies. Patients must adhere to the following:

- Patients will be instructed not to take any additional medications (including over-the-counter products) during the course of the study without prior consultation with the investigator. At each visit, the investigator will ask the patient about any new medications he/she is or has taken after the start of the study drug.
- All Concomitant medications/Significant non-drug therapies taken within 28 days of starting study treatment through the 30- day safety follow up visit, including physical therapy and blood transfusions, should be recorded.
- Patients on chronic medications that can be given concomitantly with the study agents should be continued. The investigator should instruct the patient to notify the study site about any new medications he/she takes after the start of the study drug. All medications (other than study drug) and significant non-drug therapies (including physical therapy and blood transfusions) administered after the patient starts treatment with study drug, and any changes in dosing should be recorded.
- All supportive measures consistent with optimal patient care can be given throughout the study at the discretion of the treating physician, as long as they are not part of the list of prohibited medications. In general, the use of any concomitant medication/therapies deemed necessary for the care of the patient is permitted with the following considerations:
- Prophylactic anti-emetics should be started only once the patient experienced nausea or vomiting at the discretion of the investigator. It is recommended that patients use drugs that do not cause QT prolongation.
- Patients may not receive radiotherapy while on this trial.
- Other investigational therapies must not be used while the patient is on the study

- Anticancer systemic therapy other than the study treatments must not be given to patients while on the study. If such agents are required for a patient then the patient must be discontinued from the study.
- Herbal preparations are not allowed throughout the study. These herbal preparations include, but are not limited to: St. John's wort, Kava, ephedra (ma huang), gingko biloba, dehydroepiandrosterone (DHEA), yohimbe, saw palmetto, and ginseng. Patients should stop using these herbal preparations 14 days prior to first dose of study drug

7.6 Drug-Drug Interactions

The following restrictions apply during the entire duration of the study:

- No other investigational therapy should be given to patients.
- No anticancer agents other than the study medication should be given to patients. If such agents are required for a patient then the patient must first be withdrawn from the study.
- Hydroxychloroquine interacts with CYP enzymes, causing potential drug-drug Interations. CYP3A4 modifiers are described below (Table 3) and must be evaluated carefully. Examples are shown in the table. A comprehensive list of cytochrome P450 isoenzymes and CYP3A4 inhibitors, inducers, and substrates can be found at http://medicine.iupui.edu/flockhart. This website is continually revised and should be checked frequently for updates.
- Co-administration with strong inhibitors of CYP3A4 (e.g., ketoconazole, itraconazole, ritonavir) or P-glycoprotein (PgP) should be avoided. Seville orange, star fruit, grapefruit and their juices affect P450 and PgP activity. Concomitant use should be avoided
- Co-administration with moderate CYP3A4 inhibitors (e.g., erythromycin, fluconazole) or PgP inhibitors should be used with caution. If patient requires co-administration of moderate CYP3A4 inhibitors or PgP inhibitors, reduce the dose of EVE to 5 mg daily. Additional dose reductions to 5 mg every other day may be required to manage toxicities. If the inhibitor is discontinued the EVE dose should be returned to the dose used prior to initiation of the moderate CYP3A4/PgP inhibitor.
- No chronic treatment with systemic steroids or other immunosuppressive agents (at a dose equivalent of greater than 20 mg prednisone per day) or other immunosuppressive agents) is allowed. Topical or inhaled corticosteroids are allowed.
- For hydroxychloroquine, there are known drug interactions with the following medications: penicillamine, telbivudine, botulinum toxins, digoxin, metoprolol, aurothioglucose, and propafenone. Depending on the specific drug, HCQ increases the levels of these other drugs, or interferes with the activity of the agent (specifically with botulinum toxin) and the combination should be used with caution.

Table 3: Partial List of Prohibited Medications Based on CYP Metabolism

Substrates (competitive inhibition)	
Antibiotics[1]: clarithromycin* erythromycin	Calcium Channel Blockers: amlodipine diltiazem

telithromycin* Anti-arrhythmics: quinidine Benzodiazepines: alprazolam diazepam midazolam triazolam Immune Modulators: cyclosporine tacrolimus (FK506) HIV Protease Inhibitors: indinavir* ritonavir* saquinavir* Prokinetic: cisapride Antihistamines: astemizole chlorpheniramine45	felodipine nifedipine nisoldipine nitrendipine verapamil HMG CoA Reductase Inhibitors[2]: atorvastatin cerivastatin lovastatin simvastatin Miscellaneous: aprepitant buspirone haloperidol methadone pimozide quinine sildenafil tamoxifen trazodone vincristine
Inducers	
Carbamazepine Phenobarbital Phenytoin* Rifabutin*	Rifampin* St John's wort Troglitazone
Inhibitors	
Amiodarone Cimetidine Clarithromycin Delaviridine Diltiazem Erythromycin Fluvoxamine* Grapefruit juice Sevilla orange	Indinavir Itraconazole* Ketoconazole* Voriconazole* Posaconazole* Mibefradil Nefazodone* Nelfinavir* Troleandomycin Verapamil

Based on: Ingelman-Sundberg M, Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms, Naunyn Schmiedebergs Arch Pharmacol. 2004 Jan;369(1):89-104. and [http://www.medicine.iupui.edu/flockhart/clinlist.htm as of July 13, 2006]

* asterisk denotes strong inhibition/ induction

Please note:

strong inhibitor implies that it can cause ≥5-fold increase in AUC or ≥80% decrease in clearance of sensitive CYP substrates

moderate inhibitor implies that it can cause 2 to 5-fold increase in AUC values or 50-80% decrease in clearance of sensitive CYP substrates.

(Distinction is not always categorical as interaction can vary according to conditions).

Macrolide antibiotics: Azithromycin is not a CYP3A substrate. It may therefore be employed where antibiotherapy with a macrolide is desirable in a patient being treated with EVE

Statins: Atorvastatin and pravastatin may be associated with EVE, since a PK interaction study has shown that there is no relevant PK interaction.

7.7 Criteria for Removal from Study

Patients may be removed from the study before completing the full follow-up

period in the event of unacceptable toxicity, or either physician or patient preference.

7.8 Discontinuation of Treatment

Patients who discontinue study treatment prior the final planned dose will enter the follow up phase, and complete all procedures and follow-up dictated by the protocol. Patients who choose to discontinue all study participation, including follow-up, may do so at any time without penalty, by following the procedures outlined in section 7.11. At the time of any treatment discontinuation or withdrawal, site research staff should document the reasons and conditions under which discontinuation of treatment occurred and whether the patient is willing to continue follow up or will be requesting full withdrawal from the study. Each subject has the right to discontinue study treatment or withdraw from the overall study at any time without prejudice. The investigator may discontinue any subject's participation for any reason, including adverse event or failure to comply with the protocol. Because this is a randomized pilot trial following intent-to-treat principals, patients who discontinue study treatment will be followed for study outcomes as outlined in the protocol. Reasons for withdrawal from study treatment include the following:

- Delay in treatment > 28 days due to treatment-related toxicity or major event (if due to treatment-related toxicity, patient will be taken off the treatment that is most likely to be related to the toxicity).
- Disease Recurrence: Remove patient from protocol therapy at the time progressive disease is documented.
- Extraordinary Medical Circumstances: If at any time the treating physician feels constraints of this protocol are detrimental to the patient's health remove the patient from protocol therapy.
- Patient's refusal to continue treatment: In this event, document the reason(s) for withdrawal.
- Failure to comply with protocol (as judged by the investigator such as compliance below 80%, failure to maintain appointments, etc.).

7.9 Withdrawal from Study

Patients who choose to withdraw from study participation (including any additional treatment, follow-up, or study procedures) may do so, without penalty, by sending a request to be withdrawn from the study, in writing, to the Principal Investigator. In addition, this request should specifically address whether research samples that remain in the possession of the study staff can be used for future research per the previously signed consent form. Should the patient request withdrawal from the study, the PI and project manager should be alerted immediately, and updated with the conditions of the request.

7.10 Contraceptive Measures and Reproductive Risks

Female Participants

Female participants should not become pregnant while on this study and for 8 weeks after their last dose of study treatment because the study drugs could have a negative effect on an unborn baby. In addition, female participant should not breastfeed while on this study during treatment and for 2 weeks after the last dose

as these drugs may also affect a breast-feeding child. Pregnant women and women who are breast-feeding are not allowed to participate in this study. If a participant becomes pregnant, the participant will no longer be able to participate in this study.

If a participant is of child bearing potential, she must agree to use two medically accepted forms of birth control including condoms, diaphragms, cervical cap, an intra-uterine device (IUD), surgical sterility (tubal ligation or a partner that has undergone a vasectomy), or oral contraceptives, OR she must agree to completely abstain from intercourse during participation in this study and for 8 weeks after her last dose of study treatment. Abstinence at certain times of the cycle only, such as during the days of ovulation, after ovulation and withdrawal are <u>not</u> acceptable methods of birth control. The participant's form of birth control must be approved by the study doctor.

Even when approved contraceptive methods are used, there is always a small risk that a participant could still become pregnant. If a participant becomes pregnant during the course of this study or up to 8 weeks after the last dose of study treatment, the participant must discontinue study treatment, tell the investigator immediately, and consult an obstetrician or maternal-fetal specialist. If a participant becomes pregnant while on this study, the study staff will ask permission to collect information about the participant's pregnancy.

<u>Male Participants</u>

Male participants should not father a child while on this study and for 8 weeks after the last dose of study treatment, because the drug involved could have a negative effect on an unborn baby. If a participant's spouse or partner has the potential to become pregnant, both the participant and partner must use two medically accepted forms of birth control including condoms, diaphragms, cervical cap, an intra-uterine device (IUD), surgical sterility (vasectomy or a partner that has undergone a tubal ligation), or oral contraceptives, OR the participant must agree to completely abstain from intercourse during participation in this study and for 8 weeks after the last dose of study treatment. Abstinence at certain times of the cycle only, such as during the days of ovulation, after ovulation and withdrawal are not acceptable methods of birth control. The participant's form of birth control must be approved by the study doctor.

Male participants should also inform their partner of the potential harm to an unborn child. If a participant's partner should become pregnant during the course of this study or up to 8 weeks after the participant's last dose of study treatment, the pregnancy will need to be reported to the study doctor immediately, and the participant's partner should promptly notify her doctor. The study doctor will also ask to follow-up on the pregnancy.

<u>Fertility</u>

The potential for everolimus to cause infertility in male and female patients is unknown. However, menstrual irregularities, secondary amenorrhea and associated luteinizing hormone (LH)/follicle stimulating hormone (FSH) imbalance has been

observed in female patients receiving everolimus. Blood levels of FSH and LH increased, blood levels of testosterone decreased, and azoospermia have been observed in male patients receiving everolimus.

8 EXPECTED TOXICITIES AND DOSING DELAYS/DOSE MODIFICATIONS

8.1 Toxicity Criteria

This study will utilize the CTCAE version 4.0 for toxicity and Adverse Event Reporting. A copy of the CTCAE version 4.0 can be downloaded from the CTEP home page (http://ctep.cancer.gov). All appropriate treatment areas should have access to a copy of the CTCAE version 4.0

8.2 Anticipated Toxicities of Hydroxychloroquine

Toxicities that can be seen with hydroxychloroquine include:

- Central nervous system: Irritability, nervousness, emotional changes, nightmares, psychosis, headache, dizziness, vertigo, seizure, ataxia, lassitude.
- Dermatologic: Bleaching of hair, alopecia, pigmentation changes (skin and mucosal; black-blue color), rash (urticarial, morbilliform, lichenoid, maculopapular, purpuric, erythema annulare centrifugum, Stevens-Johnson syndrome, acute generalized exanthematous pustulosis, and exfoliative dermatitis).
- Gastrointestinal: Anorexia, nausea, vomiting, diarrhea, abdominal cramping.
- Hematologic: Aplastic anemia, agranulocytosis, leukopenia, thrombocytopenia, hemolysis (in patients with glucose-6-phosphate deficiency).
- Hepatic: Abnormal liver function/hepatic failure (isolated cases).
- Neuromuscular & skeletal: Myopathy leading to progressive weakness and atrophy of proximal muscle groups (may be associated with mild sensory changes, loss of deep tendon reflexes, and abnormal nerve conduction).
- Ocular: Disturbance in accommodation, keratopathy, corneal changes/deposits (visual disturbances, blurred vision, photophobia - reversible on discontinuation), macular edema, atrophy, abnormal pigmentation, retinopathy (early changes reversible - may progress despite discontinuation if advanced), optic disc pallor/atrophy, attenuation of retinal arterioles, pigmentary retinopathy, scotoma, decreased visual acuity, nystagmus.

8.3 Anticipated Toxicities of Everolimus

Toxicities that can be seen with Everolimus include:

- Cardiovascular: Edema, peripheral edema, chest pain, congestive cardiac failure
- Central nervous system: Pain, fever, headache, insomnia, dysgeusia, ageusia
- Dermatologic: Rash, pruritus, nail disorder/thinning, dry skin, acne, erythema, hand-foot syndrome, angioedema
- Endocrine & metabolic: decreased appetite, Hyperglycemia, hypercholesterolemia, hypertriglyceridemia, hyperlipidemia, hypophosphatemia, hypokalemia, diabetes mellitus, dehydration
- Gastrointestinal: Mucositis, nausea, anorexia, diarrhea, abdominal pain, constipation , stomatitis, taste disturbance, vomiting, weight loss, dry mouth, dyspepsia, dysphagia
- Genitourinary: Urinary tract infection
- Hematologic: Anemia, lymphopenia, thrombocytopenia (DLT), leukopenia, neutropenia, pancytopenia, pure red cell aplasia
- Vascular disorders: hemorrhage, hypertension, deep vein thrombosis
- Hepatic: Alkaline phosphatase increased, AST increased
- Neuromuscular & skeletal: Weakness, back pain, arthralgia
- Renal: Creatinine increased, proteinuria, renal failure
- Respiratory: Dyspnea, cough, epistaxis, pharyngitis, pneumonitis, hemoptysis, pulmonary embolism, acute respiratory distress syndrome
- Miscellaneous: Infection (includes abscess, bronchitis, cellulitis, herpes simplex, herpes zoster), hypersensitivity, amenorrhea, aspartate aminotransferase increased alanine aminotransferase increased
- Other Medical Problems: The presence of other medical problems may affect the use of this medicine. For example bowel problems, diabetes, hyperlipidemia (increased blood cholesterol or fats), infection, kidney disease, lung disease (This medicine may worsen these conditions, or brain tumor (Use with caution). This medicine may increase the risk of having serious bleeding in the head.

8.4 Monitoring of EVE suspected toxicities

Patients whose treatment is interrupted or permanently discontinued due to

an adverse event or abnormal laboratory value suspected to be related to EVE must be followed at least weekly until the adverse event or abnormal laboratory resolves or returns to grade ≤1. If a patient requires a dose delay of > 28 days from the intended day of the next scheduled dose, then the patient must be discontinued from the study or continued on HCQ alone if there is evidence of clinical benefit and toxicities resolve to < grade 2.

8.5 Special Toxicity Considerations for EVE

8.5.1 Hyperlipidemia

Hyperlipidemia was reported as a serious adverse reaction. It is a recognized side-effect of rapamycins. Use of lipid-lowering drugs should be associated with dietary recommendations. If blood lipid levels exceed the thresholds identified in eligibility criterion 4.2.13, then fasting blood lipid levels will be assessed. As long as blood lipid levels remain below the thresholds in 4.2.13, monitoring of blood lipid levels can continue with patient in a non-fasting state. However, if at any time blood lipid levels exceed these thresholds, further monitoring of blood lipid levels requires patients to be fasting. Fasting status must be verified when interpreting results. For guidance on management of hyperlipidemia refer to Section 8.6.5.

8.5.2 Hyperglycemia

Hyperglycemia was reported as a serious adverse reaction. Similarly, the fasting state of patients should be verified when interpreting results. For guidance on management of hyperglycemia refer to Section 8.6.5.

8.5.3 Pneumonitis

Pneumonitis is a recognized adverse effect of rapamycins (sirolimus, temsirolimus, and everolimus). Numerous case reports in the literature suggest that rapamycin-associated pneumonitis is relatively unaggressive, limited in extent, and reversible upon drug discontinuation. The term ‘pneumonitis’ is used here to describe non-infectious, non-malignant infiltration in the lungs which is evident radiologically. More precise diagnosis should follow histocytological examination following lung biopsy, generally during bronchoscopy which may or may not be symptomatic. Advice on the management of pneumonitis has been provided in Section 8.6.6.

In oncology studies with EVE, severe pneumonitis suspected as drug-related has been reported as a serious adverse event on 13 occasions and additionally in the following associated preferred terms including acute respiratory distress syndrome (n=2), alveolitis (n=1) and allergic alveolitis (n=1), interstitial lung disease (n=10), lung infiltration (n=23), cryptogenic organizing pneumonia, lung consolidation, pulmonary alvealoar haemorrhage, pulmonary toxicity and pulmonary fibrosis (n=1, each). One fatal case of drug-related pneumonitis was reported for a patient with metastatic infiltrating ductal carcinoma of the breast treated with 10 mg/day, which developed approximately two months after starting EVE. Cytology for both the pleural and pericardial fluids was positive for malignancy. The death was considered possibly related to the underlying late stage tumor and study drug. Additionally, one patient treated with 10 mg/day died due to severe acute respiratory distress syndrome and septic shock. Thoracic CT scan demonstrated condensation

in the majority of the left lower lobe and frosted glass appearance in the left upper lobe, lingula, and right lung.

Along with the cases of non-infectious pneumonitis, serious **opportunistic infections** have also been reported in cancer patients treated with EVE: mycobactrium, aspergillus, and fatal candidal sepsis, and fatal pneumocystis carnii in particular. Because EVE, as other rapamycins, inhibits proliferation of activated lymphocytes and reduces neutrophil counts, treatment with EVE must be considered as predisposing patients to the risk of infection. This risk will be higher in patients severely immunocompromised because of their underlying disease and/or co-medications. Outcome may be fatal in case of serious infections.

8.5.4 Myelosuppression

Myelosuppresion is frequent when EVE therapy is initiated. Without clinical significance and infrequently, anemia and thrombocytopenia have been reported. In heavily pretreated patients with aggressive lymphoma, the incidence of grade 3 anemia, neutropenia, and thrombocytopenia was reported to be 11%, 16%, and 30%, respectively. Serious, suspected drug-related hemorrhages have been exceptional. Nevertheless, EVE should be considered as predisposing patients to hemorrhage, potentially fatal, should they develop severe drug-related thrombocytopenia.

8.5.5 Liver Function Abnormalities

Discrete, reversible changes in liver enzymes have been found to occur in numerous patients during treatment with EVE in oncology clinical studies, and in a study in rheumatoid arthritis. In oncology studies, these changes may be evident only in patients without severe underlying morbidity. The increase in transaminase's (AST and ALT) generally appears after 4 weeks of treatment. In all but a few cases it does not exceed Grade 1 (≤ 3.0 x ULN). Similarly, mild increases in alkaline phosphatases can coexist. Spontaneous corrections or intermittent correction with continued treatment can occur. Serum bilirubin is not increased. In studies of patients with advanced cancers, clinically relevant changes in liver enzymes have been invariably associated with the presence of liver metastases and/or progression of the underlying cancer.

8.5.6 Renal Insufficiency

Renal failure has been reported in five suspected cases to date. One patient with no alternative explanation made a complete recovery following study drug adjustment and no treatment/therapy for the event. The rest or the patients had concurrent morbidities, which might have contributed to the reported events.

8.5.7 Hypophosphatemia, hypomagnesemia, hyponatremia and hypocalcemia

These electrolyte abnormalities have been reported as serious adverse reactions. Electrolytes should be monitored in patients treated with EVE.

8.6 Management of Treatment-Related Toxicities

8.6.1 Mucosal Inflammation and Stomatitis:

Stomatitis mostly occurs within the first 8 weeks of treatment. In a single arm study in 92 postmenopausal breast cancer patients, a topical alcohol-free corticosteroid oral solution was administered as a mouthwash during the initial 8 weeks of starting treatment with Afinitor ® plus exemestane. In this study, a clinically meaningful reduction in the incidence and severity of stomatitis was observed.

Stomatitis/oral mucositis/mouth ulcers due to EVE should be treated using local supportive care. Please note that investigators in earlier trials have described the oral toxicities associated with EVE as mouth ulcers, rather than mucositis or stomatitis. If your examination reveals mouth ulcers rather than a more general inflammation of the mouth, please classify the adverse event as such. Please follow the paradigm below for treatment of stomatitis/oral mucositis/mouth ulcers:

All patients randomized to EVE should be instructed to do the following, starting one day prior to first dose:

- Maintain good oral hygiene, avoidance of spicy, acidic, hard and hot food and beverages
- Use mild-flavored toothpastes
- Use Alcohol-free steroid-based Mouthwash 10 mL (0.5 mg/5 mL dexamethasone oral solution) swish for 2 mins and spit QID

For mild toxicity (Grade 1), use conservative measures such as **non-alcoholic mouth wash or salt water (0.9%) mouth wash** several times a day until resolution.

For more severe toxicity (Grade 2 in which case patients have pain but are able to maintain adequate oral alimentation, or Grade 3 in which case patients cannot maintain adequate oral alimentation), the suggested treatments are **topical analgesic mouth treatments (i.e., local anesthetics such as benzocaine, butyl aminobenzoate, tetracaine hydrochloride, menthol, or phenol)** with or without **topical corticosteroids,** such as triamcinolone oral paste 0.1% (Kenalog in Orabase®).

Agents containing hydrogen peroxide, iodine, and thyme derivatives may tend to worsen mouth ulcers. It is preferable to avoid these agents. Antifungal agents must be avoided unless a fungal infection is diagnosed. In particular, systemic imidazole antifungal agents (ketoconazole, fluconazole, itraconazole, etc.) should be avoided in all patients due to their strong inhibition of EVE metabolism, thereby leading to higher EVE exposures. Therefore, topical antifungal agents are preferred if an infection is diagnosed. Similarly, antiviral agents such as acyclovir should be avoided unless a viral infection is diagnosed.

Note: Stomatitis/oral mucositis should be appropriately graded using the functional grading given on the NCI-CTC for adverse events, version 4.0.

8.6.2 Rash

For >Grade 1:

- Use fragrance free moisturizing cream or ointment BID over entire body.
- Use ammonium lactate 12% cream or salicylic acid 6% cream BID over dry/scaly/hyperkeratotic areas such as palms and soles.
- Use zinc oxide 13-40% at night for areas with fissures.

For > Grade 2:

- Use topical steroid ointment or cream (Clobetasol 0.05%, Betamethasone 0.05%, Fluocinonide 0.05%) BID and zinc oxide 13-40% at night for areas with fissures.

8.6.3 Nausea/Vomiting

Routine use of prophylactic anti-emetics is NOT recommended.

For Grade ≥ 2:

- Begin maximum supportive care and adequate anti-emetic treatments, including ondansetron and aprepitant. May opt to omit if grade I.

8.6.4 Diarrhea

Antidiarrheal medication per standard practice is recommended at the first sign of abdominal cramping, loose stools or overt diarrhea.

At first bout of diarrhea:

- Initiate loperamide: initial dose of 4 mg (2 tablets/capsules) with the first bout of diarrhea followed by 2 mg (1 tablet/capsule) every 4 hours or after every unformed stool (maximum 16 mg a day) and continue loperamide at this frequency until diarrhea free for 12 hours.

For persistent Grade 1 diarrhea on loperamide:

- Add Lomotil (diphenoxylate hydrochloride and atropine sulfate) 1 tablet (2.5 mg) every 6 hours to 8 hours

For persistent Grade 2:

- Consider adding octreotide (short-acting) 150 µg SC TID; or after initial dose of short-acting octreotide, if well tolerated, a single dose of octreotide LAR 20 mg IM

For Grade 3:

- Administer octreotide (100-150 µg SC BID or IV (25-50 µg/h) if dehydration is severe, with dose escalation up to 500 µg SC TID).
- Use IV fluids as appropriate.
- Consider prophylactic antibiotics as needed (eg, fluoroquinolones) especially if diarrhea is persistent beyond 24 hours or there is fever or Grade 3-4 neutropenia.

8.6.5 Management of hyperlipidemia and hyperglycemia

Treatment of hyperlipidemia should take into account the pre-treatment status and dietary habits. If blood lipid levels exceed the thresholds identified in eligibility

criterion 4.2.13 and treatment of hyperlipidemia is being considered, further blood tests to monitor hyperlipidemia must be taken in the fasting state. Hypercholesterolemia (> 300 mg/dL or 7.75 mmol/L) or hypertriglyceridemia (>2.5 x ULN) should be treated with a 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase inhibitor (e.g., atorvastatin, pravastatin) or appropriate lipid-lowering medication, in addition to diet. Patients should be monitored clinically and through serum biochemistry for the development of rhabdomyolysis and other adverse events as required in the product label/data sheets for HMG-CoA reductase inhibitors.

Note: Concomitant therapy with fibrates and an HMG-CoA reductase inhibitor is associated with an increased risk of a rare but serious skeletal muscle toxicity manifested by rhabdomyolysis, markedly elevated creatine kinase (CPK) levels and myoglobinuria, acute renal failure and sometimes death. The risk versus benefit of using this therapy should be determined for individual patients based on their risk of cardiovascular complications of hyperlipidemia.

Grade 3 **hyperglycemia** has been observed in patients receiving EVE therapy. In many cases in study EVE C2222, the affected patients had an abnormal fasting glucose at baseline. Based on this finding, it is suggested that optimal glucose control should be achieved before starting a patient on EVE and should be monitored during EVE therapy.

8.6.6 Management of non-infectious pneumonitis

Both asymptomatic radiological changes (grade 1) and symptomatic non-infectious pneumonitis (grade 2 = not interfering with activities of daily living or grade 3 = interfering with activities of daily living and oxygen indicated) have been noted in patients receiving EVE therapy. Non-infectious pneumonitis has been associated with EVE and other mTOR inhibitors (Atkins 2004). In order to monitor for asymptomatic (grade 1) pulmonary infiltrates, a chest X-ray is required if a CT scan of chest is not used for bi-monthly disease evaluations. Additional chest CT scans may be performed, when clinically necessary. If non-infectious pneumonitis develops, a consultation with a pulmonologist should be considered. If the patient develops grade 3 pneumonitis, treatment with EVE should be interrupted and the patient should be treated as medically indicated (short course corticosteroids, oxygen, etc).

Management of non-infectious pneumonitis suspected to be associated with EVE and dose modifications instructions are provided below.

Table 4: Management of non-infectious pneumonitis

Worst Grade Pneumonitis	Required Investigations	Management of Pneumonitis	EVE Dose Adjustment
Grade 1	**CT scans with lung windows and pulmonary function testing including: spirometry, DLCO, and room air O_2 saturation at rest. Repeat chest x-ray/CT scan every 2 Cycles until return to baseline.**	**No specific therapy is required**	**Administer 100% of EVE dose.**
Grade 2	**CT scan with lung windows and pulmonary function testing including: spirometry, DLCO, and room air O_2 saturation at rest. Repeat each subsequent Cycle until return**	**Symptomatic only. Prescribe corticosteroids if cough is troublesome.**	**Reduce EVE dose until recovery to ≤ Grade 1. EVE may also be interrupted if symptoms are troublesome. Patients will be withdrawn from the study if they fail to**

Worst Grade Pneumonitis	Required Investigations	Management of Pneumonitis	EVE Dose Adjustment
	to baseline. Consider bronchoscopy *		**recover to ≤ Grade 1 within 3 weeks.**
Grade 3	**CT scan with lung windows and pulmonary function testing including: spirometry, DLCO, and room air O_2 saturation at rest.; Repeat each subsequent Cycle until return to baseline. Bronchoscopy is recommended ***	**Prescribe corticosteroids if infective origin is ruled out. Taper as medically indicated.**	**Hold treatment until recovery to ≤ Grade 1. May restart protocol treatment within 2 weeks at a reduced dose (by one level) if evidence of clinical benefit. Patients will be withdrawn from the study if they fail to recover to ≤ Grade 1 within 2 weeks.**
Grade 4	**CT scan with lung windows and required pulmonary function testing includes: spirometry, DLCO, and room air O_2 saturation at rest. Repeat each subsequent Cycle until return to baseline. Bronchoscopy is recommended *.**	**Prescribe corticosteroids if infective origin is ruled out. Taper as medically indicated.**	**Discontinue treatment.**

***A bronchoscopy with biopsy and/or bronchoalveolar lavage is recommended.**

8.6.7 Wound healing complications

Impaired wound healing is a class effect of rapamycin derivatives, including everolimus. Caution should therefore be exercised with the use of Afinitor in the peri-surgical period.

8.6.8 Management of hypophosphatemia

Hypophosphatemia is a known side effect of EVE and will be monitored for patients receiving EVE. The CTCAE v4 grading of hypophosphatemia appears to be incompatible with the HUP laboratory's reference range. The HUP lab's LLN is 2.4 mg/dL. Grade 1 is <LLN – 2.5 mg/dL (which is N/A), Grade 2 is <2.5 – 2.0 mg/dL, Grade 3 is <2.0 – 1.0 mg/dL. Thus, a subject's phosphorus could be just 0.1 mg/dL below normal be considered a Grade 2 by the CTCAE v4. Therefore, while this protocol will still use the CTCAE v4 for grading purposes, we have instituted the guidelines outlined below for the management of hypophosphatemia. (It is noted that the CTCAE v5 has revised the hypophosphatemia grading guidelines.)

Hypophosphatemia should be managed with supplementation/oral replacement therapy as clinically indicated, at the discretion of the PI/treating physician. Events of hypophosphatemia, even if Grade 3 by the CTCAE v4 will not be subject to the EVE dose modification guidelines for non-hematological toxicity outlined in Table 11. EVE should be interrupted only if the hypophosphatemia event is severe or medically significant as determined by the PI/treating physician and/or if hospitalization or prolongation of existing hospitalization is indicated (these are the Grade 3 criteria in CTCAE v5).

8.6.9 Management of Hepatitis reactivation/flare

In cancer patients with hepatitis B, whether carriers or in chronic state, use of antivirals during anticancer therapy has been shown to reduce the risk of hepatitis B virus (HBV) reactivation and associated HBV morbidity and mortality (Loomba et al.

2008).

8.6.9.1 Monitoring and prophylactic treatment for hepatitis B reactivation

Table 5 provides details of monitoring and prophylactic therapy according to the baseline results of viral load and serologic markers testing.

Table 5: Action to be taken for positive baseline hepatitis B results

Test	Result	Result	Result	Result	Result
HBV-DNA	+	+ or -	-	-	-
HBsAg	+ or -	+	-	-	-
HBs Ab	+ or -	+ or -	+ and no prior HBV vaccination	+ or -	- or + with prior HBV vaccination
HBc Ab	+ or -	+ or -	+ or -	+	-
Recom-mendation	Prophylaxis treatment should be started 1-2 weeks prior to first dose of study drug Monitor HBV-DNA approximately every 6 weeks		No prophylaxis Monitor HBV-DNA approximately every 3 weeks		No specific action

Antiviral prophylaxis therapy should continue for at least 4 weeks after last dose of study drug. For hepatitis B reactivation, definition and management guidelines, see Table 6.

Table 6: Action to be taken in event of Hepatitis Reactivation

HBV reactivation (with or without clinical signs and symptoms)*	
For patients with baseline results: Positive HBV-DNA OR positive HBsAg -------------------------------------- reactivation is defined as: [Increase of 1 log in HBV-DNA relative to baseline HBV-DNA value OR new appearance of measurable HBV-DNA]	Treat: Start a second antiviral AND Interrupt study drug administration until resolution: ≤ baseline HBV-DNA levels If resolution occurs within ≤ 28 days, study drug should be re-started at one dose lower, if available. If the patient is already receiving the lowest dose of study drug according to the protocol, the patient should restart at the same dose after resolution. Both antiviral therapies should continue at least 4 weeks after last dose of study drug. If resolution occurs > 28 days, patients should discontinue study drug but continue both antiviral therapies at least 4 weeks after last dose of study drug.
For patients with baseline results: Negative HBV-DNA and HBsAg AND [Positive HBs Ab (with no prior history of vaccination against HBV), OR positive HBc Ab] ------------------------------------- reactivation is defined as: New appearance of measurable HBV-DNA	Treat : Start first antiviral medication AND Interrupt study drug administration until resolution: ≤ baseline HBV-DNA levels If resolution occurs within ≤ 28 days study drug should be re-started at one dose lower, if available. If the patient is already receiving the lowest dose of study drug according to the protocol, the patient should restart at the same dose after resolution. Antiviral therapy should continue at least 4 weeks after last dose of study drug.

	If resolution occurs > 28 days, patients should discontinue study drug but continue antiviral therapy at least 4 weeks after last dose of study drug.

* All reactivations of hepatitis B are to be recorded as grade 3 (CTCAE v 3.0 Metabolic Laboratory/Other: Viral Re-activation), unless considered life threatening by the investigator; in which case they should be recorded as grade 4 (CTCAE v 3.0 Metabolic Laboratory/Other: Viral Re-activation). Date of viral reactivation is the date on which **both** DNA and ALT criteria were met (e.g. for a patient who was HBV-DNA positive on 01-JAN-10 and whose ALT reached ≥ 5 × ULN on 01-APR-10, the date of viral reactivation is 01-APR-10).

8.6.9.2 Monitoring for Hepatitis C Flare

The following two categories of patients should be monitored every 6 weeks for HCV reactivation:

1. Patients with detectable HCV RNA-PCR test at baseline.
2. Patients known to have a history of HCV infection, despite a negative viral load test at baseline (including those that were treated and are considered 'cured')

For definition of hepatitis C reactivation and the management guidelines, see Table 3-4 Guidelines for management of hepatitis C.

Table 7: Guidelines for management of hepatitis C

HCV flare*	
For patients with baseline results: Detectable HCV-RNA, HCV flare is defined as: 2 log10 IU/mL increase in HCV-RNA AND ALT elevation x 5 ULN or 3 baseline level, whichever is higher	Discontinue study drug
For patients with baseline results: Knowledge of past hepatitis C infection with no detectable HCV-RNA, HCV flare is defined as: New appearance of detectable HCV-RNA AND ALT elevation x 5 ULN or 3 baseline level, whichever is higher	Discontinue study drug

* All flares of hepatitis C are to be recorded as grade 3 (CTCAE v3.0 Metabolic Laboratory/Other: Viral Flare), unless considered life threatening by the investigator; in which case they should be recorded as grade 4 (CTCAE v 3.0 Metabolic Laboratory/Other: ViralFlare).

8.7 Dose Delays

. Treatment should be delayed for major events if HCQ or EVE may further complicate the major event. "Major events" are non treatment-related grade 3 and 4 hematologic and non-hematologic toxicities. If a major event requires a delay of treatment, treatment must be delayed until toxicity is resolved (≤ Grade 1 or ≤ Baseline). If toxicity is not resolved in ≤ 4 weeks (28 days), treatment will be discontinued (see Section 7.8).

8.8 Dose Modifications

For patients who are unable to tolerate the protocol-specified dosing

schedule, dose adjustments are permitted in order to keep the patient on study drug. Dose modification guidelines are found in the sections below. Any consideration to deviate from these protocol-defined dose modification guidelines should be discussed with the Principal Investigator and treating physician for approval or disapproval in advance. Since this is a pilot study, this protocol allows physician discretion surrounding dose adjustment decisions. Dose adjustment decisions based on PI/physician discretion must be documented as such.

Because nausea, vomiting, diarrhea or rash may be attributable to either HCQ or EVE, Table 8 outlines the order in which to dose reduce one or the other agent. All other toxicities should be managed with dose modification of EVE alone.

Table 8: Guidelines for Selecting Agent for Dose Reduction if Overlapping Toxicities

Overlapping Toxicity	Dose Reduce First	Dose Reduce Second
Rash	EVE	HCQ
Nausea	HCQ	EVE
Anorexia	HCQ	EVE
Diarrhea	EVE	HCQ

8.8.1 Hydroxychloroquine Dose Modification

Any AE of ≥ Grade 3 and attributed as possibly, probably or definitely related to HCQ will result in the dose being held until the AE has resolved to ≤ grade 1 or baseline while EVE dosing may continue uninterrupted. If the AE resolves, reinstitution of treatment can occur but at a reduced dose as described in Table 9. If the AE recurs at the reduced dose, treatment will be held until the AE has resolved to ≤ grade 1 and when resolved treatment can be reinstituted at the next lower dose level. No more than 2 dose reductions are allowed during the maintenance cycles.

Table 9: Hydroxychloroquine Dose Reduction Schema

Dose Level	Dose mg/day	Reduce to
-2	400 mg twice daily	400 mg daily
-1	600 mg am and 400 mg pm	400 mg twice daily
1	600 mg twice daily	600 mg am and 400mg pm

If any AEs occur at grade ≤ 2 that are attributed as possibly, probably or definitely related to HCQ, HCQ may be continued and the AE managed with supportive care, with the exception of visual field deficit. **Visual field deficit is a potential symptom of peripheral retinal toxicity should prompt drug discontinuation and ophthalmologic evaluation immediately. With particular regard to visual field deficits patients should be cautioned to report any visual symptoms, particularly difficulty seeing entire words or faces, intolerance to glare, decreased night vision, or loss of peripheral vision**.

If any AEs occur at grade ≤ 2 that are attributed as possibly, probably or definitely related to HCQ, HCQ may be interrupted if the toxicity is either intolerable to the patient or PI/physician discretion determines it is in the best interest of the patient. In such cases, HCQ should be interrupted until recovery to grade ≤1. If

resolution occurs in ≤ 7 days HCQ may be reintroduced at the same dose. If resolution takes > 7 days HCQ should be reintroduced at the lower dose level. If the event returns to grade 2, then HCQ should be interrupted until recovery to grade ≤1, after which HCQ should be reintroduce at the lower dose level.

8.8.1.1 Management of suspected HCQ induced myopathy

Myopathy leading to muscle weakness is a rare but potentially serious side effect of HCQ. In our experience, this toxicity resolves with holding drug and can be reduced by reducing dose. With close attention to early signs of this toxicity, the goal is for it to be managed before it becomes serious and reaches a higher grade. Therefore, in the instance of grade >1 muscle weakness and/or elevated CPK or at MD discretion (even if the related AE is still only grade 1), HCQ should be held. When myopathy/muscle weakness returns to grade ≤1 (or after holding for a period per MD discretion, as long as symptoms and lab values are trending back to normal), HCQ may be reintroduced at the next lower dose level. It is not necessary that CPK return to grade ≤1 to restart drug, so long as myopathy has. It is intended that close monitoring and implementing HCQ dose reduction at early signs of myopathy will prevent subjects progressively worsening to the point that they have to come off study drug (HCQ) altogether.

8.8.2 Everolimus (EVE) Dose Reduction Guidelines

Known EVE hematological toxicities will not be attributed to HCQ but will only be attributed to EVE and will result in the following EVE dose modifications, as shown in Table 11. Hematologic toxicity of unexpected severity may be attributed to HCQ (in addition to EVE at investigator discretion). Non-hematological toxicities having an attribution to EVE of possible, probable or definite will result in the following EVE dose modifications, as shown in Table 11. For EVE dosing, the patient must have an ANC ≥ 1.0 x 10^9/L and platelet count > 75 x 10^9/L. All non-hematological toxicity grade ≥ 3 (except for alopecia, nausea and vomiting) must have resolved to CTCAE grade ≤ 1. If toxicity persists, treatment should be delayed by one week for up to 4 consecutive weeks. If after 4 weeks (28 days) of delay all toxicity has still not resolved, any further treatment with EVE should be stopped. HCQ may be continued after EVE has been stopped on the combination arm.

Table 10: EVE Dose Reduction Guidelines

Dose Level	Dose	Reduce to
1	**10 mg daily**	**5 mg daily**
-1	**5 mg daily**	**5 mg every other day**
-2	**5 mg every other day**	**Discontinue EVE**

If any non-hematological toxicity observed Grade ≥ 3 (except for alopecia, nausea and vomiting) and/or if platelets < 75 x 10^9/L and/or ANC < 1 x 10^9/L, causing delay of two weeks of EVE then the dose should be reduced by one dose level (see Table 10). For patients who would require dose >2 dose reductions EVE will be stopped. If any non-hematological toxicity observed was CTCAE **Grade 4** (except for alopecia, nausea and vomiting) then EVE should be **stopped.**

Table 11: Criteria for dose-modification for suspected EVE toxicity and re-initiation of EVE treatment

Toxicity	Actions
Non-hematological toxicity	
Grade 2 (except pneumonitis – refer to Table 6)	If the toxicity is tolerable to the patient, maintain the same dose. If the toxicity is intolerable to patient, interrupt EVE until recovery to grade ≤1. If resolution occurs ≤ 7 days reintroduce EVE at same dose. If resolution takes > 7 days or if the event returns to grade 2, then interrupt EVE until recovery to grade ≤1. Then reintroduce EVE at lower dose level.
Grade 3 (except hyperlipidemia*) (except pneumonitis – refer to Table 6) (except hypophosphatemia, refer to Section 8.6.8)	Interrupt EVE until recovery to grade ≤1. Then reintroduce EVE at the lower dose level. For pneumonitis consider the use of a short course of corticosteroids.
Grade 4	Discontinue EVE
Hematological toxicity	
Grade 2 Thrombocytopenia (<75,000 – 50,000/mm^3 ; <75 – 50.0 x 10^9/L)	Temporary dose interruption until recovery to Grade ≤ 1. Re-initiate treatment at the same dose.
Grade 3 Thrombocytopenia (<50,000 – 25,000/mm^3 ; <50.0 – 25.0 x 10^9/L)	Interrupt EVE until resolution to grade ≤1 Re-initiate treatment at a lower dose.
Grade 4 Thrombocytopenia (<25,000/mm^3 ; <25.0 x 10^9/L)	Interrupt EVE until recovery to grade ≤ 1. Then reintroduce Everolimus at one dose level lower, if available.
Grade 3 Neutropenia (<1,000 – 500/mm^3 ; <1.0 – 0.5 x 10^9/L) or anemia	Temporary dose interruption until recovery to Grade ≤ 2. Re-initiate treatment at the same dose
Grade 4 Neutropenia (<500/mm^3 ; <0.5 x 10^9/L) or anemia	Interrupt EVE until recovery to grade ≤ 2. Reintroduce EVE at one dose level lower, if available.**
Grade 3 Febrile Neutropenia (ANC <1,000/mm^3 with a single temperature of > 38.3 °C (101°F) or a sustained temperature of ≥ 38 °C (100.4 °F) for more than one hour)	Hold further dosing until Grade ≤ 2 ANC and no fever. Then resume dosing at the next lower dose level if available.
Grade 4 Febrile Neutropenia (life-threatening consequences ; urgent intervention indicated))	Discontinue EVE.
Recurrence of grade 3 toxicity after dose reduction	Reduce dose to the next lower dose level, if available. The lowest possible dose level of EVE is 5 mg every other day (2.5 mg daily). Below this level, EVE must be discontinued.
**Recurrence of grade 4 toxicity (including febrile neutropenia) after dose reduction	Discontinue EVE
**Any toxicity requiring interruption for > 28 days	Discontinue EVE

*Grade 3 hyperlipidemia (hypercholesterolemia and/or hypertriglyceridemia) should be managed using medical therapies.

9 DRUG FORMULATION AND STORAGE

9.1 Agent #1: Hydroxychloroquine

Generic name: Hydroxychloroquine sulfate

Commercial name: Plaquenil

Chemical name: 7-Chloro-4-[4-[ethyl-(2-hydroxyethyl)amino]-1-methylbutylamino] quinolone

Hydroxychloroquine sulfate is a colorless crystalline solid, soluble in water to at least 20
percent; chemically the drug is 2-[[4-[(7-Chloro-4-quinolyl) amino]pentyl] ethylamino] ethanol
sulfate (1:1).

9.1.1 Supply

As described above, hydroxychloroquine will be obtained through a written prescription by study MD as an initial 28- day supply and refilled every cycle. Patients will be required to bring the prescription to their visit on day 1 of each cycle, along with previous months' pill container so that pill counts can be performed. In addition, treatment diaries will be used to monitor adherence to therapy. Patients are required to bring the pill bottles and treatment diaries to each visit.

9.1.2 Formulation

PLAQUENIL (hydroxychloroquine sulfate) tablets contain 200 mg hydroxychloroquine
sulfate, equivalent to 155 mg base, and are for oral administration.
Inactive Ingredients: Dibasic Calcium Phosphate, Hydroxypropyl Methylcellulose, Magnesium Stearate, Polyethylene glycol 400, Polysorbate 80, Starch, Titanium Dioxide.

9.1.3 Dosing, Administration and Storage

When taking HCQ twice daily, the two daily doses should be taken 12 hours apart as close to 9am and 9pm as possible and documented clearly on the patient calendar. Patients receiving antacids, sucralfate, cholestyramine, and/or bicarbonate should have the HCQ drug dose administered at least 1 hour before or 2 hours after these medications. Missed doses should not be made up; patient should resume planned dose at next dosing interval.

9.2 Agent #2: Everolimus

Generic name: Everolimus

Commercial name: Afinitor™

Chemical name: dihydroxy-12-[(2*R*)-1-[(1*S*,3*R*,4*R*)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]propan-2-yl]-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-azatricyclo[30.3.1.$0^{4,9}$]hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentone

9.2.1 Supply

Everolimus will be supplied by Novartis Pharmaceuticals Corporation for this trial and dispensed by the Oncologic Investigational Pharmacy at the Abramson Cancer Center of the University of Pennyslvania.

9.2.2 Formulation

EVE is formulated as tablets for oral administration of 5mg strength. Tablets are blister-packed under aluminum foil in units of 10 tablets, which should be opened only at the time of administration as drug is both hygroscopic and light-sensitive.

9.2.3 Dosing, Administration and Storage

Patients randomized to an EVE-containing arm will receive EVE as a dose of 10 mg orally daily, on a continuous basis. Patients will be instructed to take EVE in the morning, at the same time each day. EVE may be taken with or without food.

9.3 Drug Accountability

Clinical drug supply must be accounted for and patients will be asked to return all unused study drug and packaging on a regular basis, at the end of the study or at the time of study drug discontinuation. Accountability for the drug at all study sites is the responsibility of the principal investigator and designated Pharmacy representative. The investigator will ensure that the investigational drug is used only in accordance with this protocol. Drug accountability records indicating the drug’s delivery date to the site, inventory at the site, use by each patient, and destruction/disposal per institutional policy will be maintained by the clinical site. Accountability records will include dates, quantities, lot numbers, expiration dates, and patient numbers.

At the conclusion of the study, and, as appropriate during the course of the study, the investigator will return all used and unused study drug, packaging, drug labels, and a copy of the completed drug accountability ledger to Novartis.

10 SAFETY ASSESSMENTS

10.1 Adverse Events Reporting Period

Patients will be evaluated for evidence of toxicity from the time of the first dose of HCQ/EVE to 30 days after last study drug or longer in the event of residual toxicity.

10.2 Definitions

Adverse Event: An adverse event (AE) is any symptom, sign, illness or

experience that develops or worsens in severity during the course of the study. Intercurrent illnesses or injuries should be regarded as adverse events. Abnormal results of diagnostic procedures are considered to be adverse events if the abnormality:

.. results in study withdrawal
.. is associated with a serious adverse event
.. is associated with clinical signs or symptoms
.. leads to additional treatment or to further diagnostic tests
.. is considered by the investigator to be of clinical significance

Serious Adverse Event: A serious adverse event (SAE) is any adverse event, without regard to causality, that is life-threatening or that results in any of the following outcomes: death; in-patient hospitalization or prolongation of existing hospitalization; persistent or significant disability or incapacity; or a congenital anomaly or birth defect. Any other medical event that, in the medical judgment of the Principal Investigator, may jeopardize the subject or may require medical or surgical intervention to prevent one of the outcomes listed above is also considered an SAE. A planned medical or surgical procedure is not, in itself, an SAE. Also specifically excluded from this definition of SAE is any event judged by the Principal Investigator to represent progression of the malignancy under study, unless it results in death within the SAE Reporting Period.

Post-study Adverse Event: All unresolved adverse events should be followed by the investigator until the events are resolved, the patient is lost to follow-up, or the adverse event is otherwise explained. At the last scheduled visit, the investigator should instruct each patient to report any subsequent event(s) that the patient, or the patient's personal physician, believes might reasonably be related to participation in this study.

10.3 Abnormal Laboratory Values

A clinical laboratory abnormality should be documented as an adverse event if the abnormality is of a degree, typically at least grade 2 and not present as grade 1 or better at baseline, that requires active management; e.g. change of dose, discontinuation of the drug, more frequent follow-up assessments, further diagnostic investigation.

10.4 Hospitalization, Prolonged Hospitalization or Surgery

Any adverse event that results in hospitalization or prolonged hospitalization should be documented and reported as a serious adverse event unless specifically instructed otherwise in this protocol. Any condition responsible for surgery should be documented as an adverse event if the condition meets the criteria for an adverse event.

Neither the condition, hospitalization, prolonged hospitalization, nor surgery are reported as an adverse event in the following circumstances:

-Hospitalization or prolonged hospitalization for diagnostic or elective surgical procedures for a preexisting condition. Surgery should not be reported as an outcome of an adverse event if the purpose of the surgery was elective or diagnostic and the outcome was uneventful.

-Hospitalization or prolonged hospitalization required to allow efficacy

measurement for the study.

-Hospitalization or prolonged hospitalization for therapy of the target disease of the study, unless it is a worsening or increase in frequency of hospital admissions as judged by the clinical investigator.

10.5 Recording of Adverse Events

At each contact with the patient, the investigator must seek information on adverse events by specific questioning and, as appropriate, by examination. Information on all adverse events should be recorded immediately in the source document, and also in the appropriate adverse event module of the case report form (CRF). All clearly related signs, symptoms, and abnormal diagnostic procedures results should recorded in the source document, though should be grouped under one diagnosis. All adverse events occurring during the study period must be recorded. The clinical course of each event should be followed until resolution, stabilization, or until it has been determined that the study treatment or participation is not the cause. Serious adverse events that are still ongoing at the end of the study period must be followed up to determine the final outcome. Any serious adverse event that occurs after the study period and is considered to be possibly related to the study treatment or study participation should be recorded and reported immediately.

10.6 Reporting of Adverse Events and Serious Adverse Events

10.6.1 Study Chair Notification by Investigator

A serious adverse event must be reported to the Study Chair within 24 hours of a member of the study team becoming aware of the event.

Report serious adverse events by phone and facsimile to:

<u>Angela DeMichele, MD, MSCE, or via the HUP page operator</u>

At the time of the initial report, the following information should be provided:

- Study identifier
- Study Center
- Patient number
- A description of the event
- Date of onset
- Current status
- Whether study treatment was discontinued
- The reason why the event is classified as serious
- Investigator assessment of the association between the event and study treatment

FAX#: 215-662-7865

10.6.2 IRB Notification by Investigator-sponsor

Reports of all serious adverse events (including follow-up information) must be submitted to the IRB and CTSRMC (Penn) within 10 working days, according to IRB guidelines.

<u>Reporting Process to IRB at Penn</u>: Principal Investigators are encouraged to submit reports of unanticipated problems posing risks to subjects or others using the form: "Unanticipated Problems Posing Risks to Subjects or Others Including Reportable Adverse Events" via HS-ERA or a written report of the event (including a description of the event with information regarding its fulfillment of the above criteria, follow-up/resolution and need for revision to consent form and/or other study documentation) within 7 working days and the PI or her designee will enter the event into HS-ERA and VELOS for Penn and participating sites. Participating sites should follow local, institutional guidelines on Event Reporting.

For reportable deaths, the initial submission to the IRB may be made by contacting the IRB Director or designee at 215.573.1206. All deaths will be reported via HS-ERA, as well.

10.6.3 FDA Notification by Investigator-sponsor

This study is IND exempt and reporting to the FDA is voluntary using a Medwatch 3500 or via the FDA's website for voluntary reporting.

10.6.4 Abramson Cancer Center DSMC notification

All adverse events meeting the ACC'S DSMC reporting requirements outlined below will be reported to the ACC DSMC through the PennCTMS (Velos) Clinical Trial Management System AE/SAE Form.

- All Grade 3 or higher adverse events regardless of attribution or expectedness must be submitted to the ACC'S DSMC **within 10 business days** of knowledge.
- All unexpected deaths must be reported **within two business days** of knowledge. Deaths related to disease progression must clearly state that fact.
- All others deaths must be reported **within 30 days** of knowledge. Deaths of subjects greater than 90 days from the last study treatment/intervention are not reportable.

All AE/SAEs should include grade, attribution and expectedness as determined by the PI or sub-I. Only an investigator may determine the attribution and expectedness.

For attributions of "unrelated", an alternative explanation must be provided to explain to what the event is being attributed.

For studies using multiple agents in a single study, the agent to which the event is being attributed should be identified.

Every effort should be made to report an event as a diagnosis, not as a list of symptoms. Symptoms that led to the diagnosis should be included in the event description, but should not be the actual event.

10.6.5 IND SAFETY UPDATES/ALERTS

All IND Safety Updates/Alerts (sent by investigators) with a grade 3 or higher, regardless of attribution or expectedness must be submitted to the DSMC within 30 days. Reports should be sent to the DSMC for 6 months from the date the last Penn

subject was treated.

Although this protocol is IND-exempt, it is the responsibility of each PI to notify all participating investigators, in a written safety report using an IND safety report template, of any adverse event associated with the use of the drug that is both serious and unexpected, as well as any finding from tests in laboratory animals that suggest a significant risk for human subjects. Additionally, investigators are also required to identify in IND safety reports all previous reports concerning similar adverse events and to analyze the significance of the current event in light of the previous reports. Reports will be sent, via e-mail to participating sites, for review and forwarding to their internal committees, as appropriate.

SAE Definition.

10.6.6 Novartis instructions for rapid notification of serious adverse events

1. The principal investigator has the obligation to report all serious adverse events to the FDA, IRB, and Novartis Pharmaceuticals Clinical Safety and Epidemiology Department (CS&E).

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

3. All events must be reported, by FAX (888-299-4565), to Novartis Pharmaceuticals CS&E Department within 24 hours of learning of its occurrence. This includes serious, related, labeled (expected) and serious, related, unlabeled (unexpected) adverse experiences. All deaths during treatment or within 30 days following completion of active protocol therapy must be reported within 5 working days.

4. Any serious adverse event occurring after the patient has been administered one dose of study drug until 4 weeks after the patient has stopped study participation must be reported. This includes the period in which the study

protocol interferes with the standard medical treatment given to a patient (e.g. treatment withdrawal during washout period, change in treatment to a fixed dose of concomitant medication).

5. Serious adverse events occurring more than 30 days after study discontinuation need only be reported if a relationship to the Novartis study drug (or therapy) is suspected.

10.7 Internal Safety and Compliance Entities

The Data Safety and Monitoring Committee (DSMC) of the University of Pennsylvania's Abramson Cancer Center will monitor the data quality and adherence to safety rules. Additionally, the DSMC will review all safety/toxicity data for the trial and recommend trial suspension or termination as needed. Specific details of monitoring and audit frequency will be included in the Monitoring Plan, but will be at least every 6 months.

Patients will be evaluated for toxicity if they have received at least one dose of HCQ.

The timely reporting of adverse events (including toxic deaths) is required by the Food and Drug Administration. The reporting of toxicities is part of the data reporting for this study.

10.8 Medical and Research Monitoring

It is the responsibility of the Principal Investigator to oversee the safety of the study at his/her site. This safety monitoring will include careful assessment and appropriate reporting of adverse events, as noted above, as well as the construction and implementation of a site data and safety monitoring plan. Medical monitoring by an independent clinician within the Department of Medicine, Division of Hematology-Oncology will include a regular assessment of the number and type of serious adverse events on a periodic basis. Dr. Tara (Gangadhar) Mitchell is a board certified oncologist, and a member of the solid malignancies clinical trials group at UPENN. She is the PI on multiple phase I/II trials. The medical monitor will meet with Dr. DeMichele and clinical research staff quarterly. She will review adverse events, response assessments, and decisions related to modifications.

Furthermore, Dr. Mitchell will serve in the role of independent research monitor. The roles/responsibilities of the independent research monitor are outlined below. It is noted that Dr. Mitchell has no apparent conflict of interest and as the independent research monitor, she is not under the supervision of the PI or other investigators or research staff.

The research monitor:

- may discuss the research protocol with the investigators, interview human subjects, and consult with others outside of the study about the research;
- shall have authority to stop a research protocol in progress, remove individual human subjects from a research protocol, and take whatever steps are

necessary to protect the safety and well-being of human subjects until the IRB can assess the monitor's report;
- shall have the responsibility to promptly report their observations and findings to the IRB or other designated official and the Human Research Protection Office (HRPO) of the US Army Medical Research and Material Command (USAMRMC)

10.9 Study Monitoring Plan

This study will be monitored by the Principal Investigator and sub-investigators, as appropriate. Such monitoring will include at least weekly meetings of the study team to review accrual, toxicity, SAEs, dose escalations and study finding. In addition, the PI will ensure that data are completed in a timely manner and he or his designee will periodically review the data for accuracy, completeness and integrity.

10.10 Auditing and Inspecting

The investigators will permit the Cancer Center’s Administrative Director of Compliance and Auditing or her designee to review records, data and facilities at mutually agreeable times.

This study will be audited by the Department of Compliance and Monitoring (DOCM) as a high-risk study. Auditing will occur 6 months after the first subject enrolls and every six months thereafter until the study terminates. Audits will be scheduled approximately 5 weeks in advance of the audit. Lack of cooperation with scheduling will result in the study being put on hold at the participating sites until an audit is conducted. The DOCM may at its discretion, modify the audit schedule or the audit process.

10.11 Data Safety Monitoring Board

In addition to the above safeguards, an independent data safety monitoring committee, consisting of 2 medical oncologists and a member of the patient advocate team will meet with the PI and the study team monthly to review safety data, to assure that no unanticipated safety signals or risks are arising in the conduct of the study. In addition, the DSMB will review interim safety analyses for each arm after every 3 patients have been enrolled. We will implement a Bayesian toxicity monitoring approach with a target protocol-defined DLT rate of 30% as outlined in the statistical plan. This additional safeguard is to ensure that patients on this trial, with potentially curable disease, are being monitored closely.

10.12 Protocol Deviations and Exception Requests

A protocol deviation is a one time, unintentional action or process that departs from the IRB and DSMC approved study protocol, involving one incident and identified retrospectively, after the event occurred. If the impact on the protocol disrupts the study design, may affect the outcome (endpoints) or compromises the safety and welfare of the subjects, the deviation is considered “major” and must be reported to the DSMC and the IRB within 10 business days.

Deviations must be submitted if they:

- have the potential to adversely affect subject safety
- increase risks to participants

- adversely affect the integrity of the data
- violate the rights and welfare of participants
- affect the subject's willingness to participate in research

Other minor deviations should be explained in a memo to file or on a deviation log and summarized at continuing review.

If there is a planned deviation from the protocol, the situation will be submitted to the IRB and DSMC PRIOR to the event as an exception request, and approval will be sought before implementation. Exceptions to eligibility, treatment/dosing, contraindicated treatment/therapies/ interventions or safety tests for investigator-initiated treatment studies may be rejected by the ACC DSMC. Exception requests must provide strong and compelling scientific and clinical information as well as a statement explaining whether or not the protocol will be amended. If the protocol will not be amended a rationale must be provided. A protocol amendment is required if this situation is likely to recur. Study PI and Medical Monitor approval must be obtained prior to submitting an exception request.

11 MEASUREMENT OF EFFECT

For the purposes of this study, patients should be re-evaluated for response per the study calendar.

11.1 Definitions

11.1.1 Evaluable for toxicity

All patients will be evaluable for toxicity from the time of their first treatment with HCQ, EVE or both.

11.1.2 Evaluable for objective response

Only those patients who have measureable DTCs present at baseline, have received at least one cycle of therapy, and have had their disease re-evaluated at the 24 week timepoint will be considered evaluable for response. These patients will have their response classified according to the definitions stated below. We will report on both the "intent-to-treat" population and the evaluable population per the above criteria in order to fully determine whether the interventions are both feasible and showing sufficient evidence of activity to take forward to a larger phase III trial powered for relapse-free survival (RFS).

11.1.3 Measurable Disease

Patients enrolled on the CLEVER trial will have completed surgery for their primary breast tumor and be free of overt metastatic disease. Thus there is no tumor to measure for the effect of the study therapy. Rather, the goal of this trial is to eradicate minimal residual disease, in the form of disseminated tumor cells (DTCs) that are present in the bone marrow. Therefore, bone marrow DTCs constitute the "measureable" disease in this trial. A patient will be classified as having "measureable disease" if at least 1 disseminated tumor cell (DTC) is detected

in 5 ml of bone marrow aspirate by immunohistochemistry, as described in section 13.2.1.

11.2 Guidelines for Evaluation of Disease

11.2.1 Measurement Methods

Bone marrow aspirate will be used to obtain sample for assessment of DTCs. This will be performed in a standard fashion as outlined in section 13. Bone marrow will be assayed for DTCs by standard immunohistochemical assay performed under CLIA conditions in the Clinical Pathology Laboratory at the University of Pennsylvania as described in section 13.

11.2.2 Response Criteria

Response in this trial will be performed in all patients, and is defined by the change in detectable DTC number (# cell/5 ml marrow aspirate) from baseline to each timepoint. We will define “response” for each patient as a 50% or greater decrease in DTC number at each treatment time point compared to baseline. The interventional arm will be considered successful if at least 20% of patients experience this predefined response endpoint. We anticipate that the baseline variability in DTC measurement will lead to no more than a 4% response rate in the absence of treatment (null rate).The efficacy objective of this trial (a secondary objective) is to evaluate the preliminary efficacy of EVE, HCQ or the combination in reducing or eliminating breast cancer DTCs, and provide estimates of effect for future definitive trials. The primary measure of efficacy in this trial is change in the number of detectable disseminated tumor cells (DTCs) in the bone marrow of patients on study. DTCs will be detected from 5 ml of bone marrow extracted from the posterior iliac crest at baseline (pre-treatment), after 3 cycles, 6 cycles, and 12 cycles (if applicable) of study treatment, and at 6 months after the last dose of study treatment (optional).

12 STATISTICAL CONSIDERATIONS AND METHODOLOGY

12.1 Sample Size

The primary endpoint of this pilot study is feasibility. 60 patients will be enrolled on the trial. Based on the literature and our preliminary experience, we anticipate detecting BM-DTCs in 20 - 25% of the patients we screen on the PENN-SURMOUNT study (IRB# 824098/UPCC 28115), thus have planned to screen 250 - 300 patients over three years, allowing for refusals and drop-out.

12.2 Analysis Plan

12.2.1 Primary Endpoint

The phase II trial endpoint “feasibility” is based upon the overall safety and tolerability of the treatment arms, and will be defined as <u>at least</u> 11 of 15 patients within each treatment group completing 6 cycles of therapy without grade 3 or 4 toxicity. When evaluating feasibility of the combination therapy, Arm C and D may be combined for analysis, since both arms receive the same study treatment (Arm D is just a delayed start). Toxicity will be determined by NCI CTCAE v4 criteria, and categorized by system and grade. Patients who drop out before the 6-month time

point for reasons other than toxicity and not related to feasibility will be replaced. With 15 patients per group, an exact 95% confidence interval around the feasibility and toxicity rates will be no more than 0.52 units wide. Arms that are discontinued for lack of feasibility will be dropped from the trial, but others may continue, so long as they remain "feasible" by this definition.

Because this is a potentially curable patient population, we will also institute an early stopping rule, should the toxicity rates exceed those expected or acceptable in this setting. To do this, we will implement a Bayesian toxicity monitoring approach with a target protocol-defined DLT rate of 30%. We assume a *Beta(1,2)* prior, equivalent to one DLT observed in 3 treated patients. Early termination for toxicity will be considered based on a posterior probability that the toxicity rate exceeds 30%. Early termination will be considered if DLT is observed in 2 of 3 patients (0.84 posterior probability), 3 of 6 patients (0.81), 4 of 9 patients (0.79), 5 of 12 patients (0.78), 6 of 15 patients (0.78).

12.2.2 Secondary Endpoints

The secondary endpoints are tolerability/toxicity and the measured effect of the investigational therapy on DTCs in patients with measureable DTCs at baseline and the comparison of this effect to that in a control population. Because this is proof-of-concept, sample sizes are not sufficient to address long-term recurrence risk. Randomization is being performed to minimize uncontrolled confounding.

We will define "response" for each patient as a 50% or greater decrease in DTC number at each treatment time point compared to baseline. A treatment arm will be considered successful if at least 3 of 15 patients experience this predefined response endpoint. This strategy is being used to provide preliminary estimates of effect on DTCs and to assess whether one or more treatment approaches has sufficient activity to be considered in a larger study powered for relapse-free survival. We anticipate that the baseline variability in DTC measurement will lead to no more than a 4% response rate in the absence of treatment (null rate). While the trial is powered for feasibility, 15 patients provide approximately 80% power to detect a response rate of 20%, warranting further study of the treatment strategy evaluated in this patient population and clinical setting.

We will also estimate, within each treatment group, the % change in BM-DTC number, defined as the difference between BM-DTCs enumerated at 6 months and at baseline, divided by the number at baseline; we will compare this quantity across treatment groups using simple regression analyses with indicator variables for the three active treatments compared to the delayed treatment. While the trial is powered for feasibility, rather than detection of change in BM-DTCs across treatment group, 15 patients per group will provide 80% power (using a two-group t-test with a two-sided alpha level of 0.05) to detect a decrease in BM-DTCs of approximately 1 standard deviation between any two groups and will provide important preliminary data for designing future trials.

Because of tumor heterogeneity, the natural history of recurrence varies substantially between breast cancer subtypes. We have elected not to expand the

sample size to evaluate subtypes individually in this feasibility pilot, but expect that we will enroll a mix of patients across subtypes that will shed light on variability in DTCs by subtype in an exploratory fashion.

In addition, we will estimate the 3-year relapse-free survival (RFS) of patients enrolled on the trial. Since this is a proof-of-concept and safety trial, we have elected not to power the trial for this endpoint, but anticipate collecting valuable information to inform and power a subsequent definitive trial powered for this endpoint.

12.2.3 Translational Endpoints

We expect these studies to inform whether gene expression features, mutations, or copy number alterations predict response of DTC numbers to treatment with HCQ, GED or their combination, including alterations in the PI3K/mTOR pathway. In addition, we anticipate that these studies will identify markers in residual tumors that could serve as therapeutic targets for future trials. In addition, all analyses involving DTC counts determined by DTC-IHC will be repeated using DTC counts from DTC-Flow and results compared between the two assays.

13 CORRELATIVE SCIENCES

Patients enrolled on the CLEVER Trial will have bone marrow aspirates and blood collected serially to perform correlative studies of DTCs, CTCs and ptDNA as per the study calendar. Tumor tissue from the primary tumor (or from residual disease after neoadjuvant therapy, if applicable) will also be obtained and subjected to whole exome sequencing (WES) to obtain mutational profiles and driver mutations to be used for ptDNA biomarkers.

13.1 Biospecimens

13.1.1 Blood samples

Blood collections will be performed at the time points depicted in the study calendar, including at the time of bone marrow aspirates and then at 6-month intervals during the follow up phase for subjects who return to Penn for their follow up visits.

13.1.2 Tissue Samples

Serial sections of FFPE-fixed primary tumors will be obtained. Samples from other institutions will be shipped to our main site. A representative top section will be H&E stained by a pathologist (Dr. Michael Feldman) to identify regions of tumor and estimate tumor cellularity. Ten unstained sections of 10um thickness will be used as starting material for simultaneous extraction of DNA and RNA. Samples with cellularity lower than 25% will undergo macro-dissection to remove non-tumor tissue prior to extraction of nucleic acid. Following deparaffinization in xylene, nucleic acids will be extracted using the AllPrep DNA/RNA FFPE kit. To assess germline variation in these patients, which is required for interpretation of NGS analyses, DNA will be extracted from buffy coat samples using Gentra Puregene Blood Kit. Nucleic acid concentrations and yield will be determined using a Qubit

fluorometer, A260/A280 ratios will be assessed using a NanoDrop spectrophotometer, and fragment length distribution and RNA Integrity Numbers (RIN) will be determined using an Agilent Bioanalyzer.

13.1.3 Bone marrow aspirates

Bone marrow aspirates will be obtained clinically within the outpatient setting, by qualified personnel. Only liquid aspirate will be collected, bone marrow core biopsies are not needed. The clinician obtaining the bone marrow sample will assess the anatomic landmarks before the area is prepped and draped in sterile fashion. The patient will be turned to a decubitus position and local anesthetic will be administered to the posterior ileac crest. Using a Jamshedi needle, approximately 10 ml of bone marrow fluid will be aspirated into two EDTA-containing tubes. 5 ml will be used for the standard immunohistochemical (IHC) assay which will be performed in the setting of the Pathology Department of the University of Pennsylvania. Additional 5 ml will be sent to the research laboratory of Dr. Lewis Chodosh at the University of Pennsylvania for DTC-FLOW analysis. Bone marrow aspirates will be divided into equal aliquots that will be used to enumerate DTCs using either DTC-Flow or DTC-IHC, and for downstream analysis. Samples will be stored at room temperature and run within 24-48 hours of aspiration.

13.2 Laboratory Assays

13.2.1 DTC Assessment by Immunohistochemistry

The presence of DTCs in the marrow by IHC is an integral biomarker required on this study for eligibility. In addition, this assay will be used to quantitate DTC response to therapy. The gold standard method for DTC assessment is immunohistochemistry (IHC), and all samples obtained during this trial will be subjected to IHC assessment. IHC to detect DTCs will be performed using the method adapted from Janni[4] and Naume[14]. This well-established assay, which has been used both in prior prognostic studies and to determine trial eligibility for additional adjuvant chemotherapy[14], has been replicated within the Research Pathology Laboratory of the University of Pennsylvania under the direction of Dr. Michael Feldman, a co-investigator on this proposal, and subsequently implemented in our CLIA laboratory for clinical use. Briefly, 5 ml of a bone marrow aspirate sample drawn from the iliac crest under anesthesia and collected in an anti-coagulated tube containing EDTA will be stored at room temperature and processed within 24 hours. Density centrifugation using Lymphoprep will be performed and mononuclear cells (MNCs) will be harvested, washed and resuspended to 2.5x10*6 cells/ml. Cytospin slides will then be prepared from the cell suspension using a cytocentrifuge and slides will be fixed in 95% alcohol before immunostaining on a Leica Bond III autostainer. An anti-pancytokeratin monoclonal antibody will be used to screen for the presence of DTCs in the bone marrow aspirate sample. Cytospin slide containing only Hs578T cancer cell line will be used as a negative control, and a positive control containing pure MCF7 cancer cell line will be included in each run together with an antibody isotype negative control. Each cytospin will be prepared with 1x10*6 MNCs and a total of 5x10*6 cells will be evaluated per patient. Cytospin slides will be reviewed by Dr. Feldman and Dr. Nayak and reported. The presence

of one or more pancytokeratin positive cells with appropriate morphology of a tumor cell will be reported as positive according to the Consensus Committee morphological criteria published by Fehm[8] and the Naume trial[14]. Additional cytospin slides will be prepared and stained in cases displaying atypical or indeterminate morphology.

13.2.2 Primary Tumor Analyses

RNA-seq, WES and copy number analysis (CNV) will be performed on all residual tumor specimens passing QC metrics. WES and CNV will be performed on DNA isolated from buffy coat samples.

RNA-seq: Libraries will be constructed using TrueSeq Stranded Total RNA Sample Prep followed by ribosomal RNA depletion using RiboZero (Gold). Libraries will be sequenced on the HiSeq4000 with 100 bp paired-end reads (100PE) for a total of 50M reads after the removal of low-quality reads. Quality of raw data will be confirmed by FASTQC. Reads will be aligned to the latest version of the human reference genome (hg38) with STAR[109] and quantified using featureCounts. D ESeq2[110,111] will be used for subsequent normalization and differential gene expression analysis.

WES: Libraries will be constructed using SureSelect v5 Library Preparation Kit. Libraries will be sequenced on the HiSeq4000 with 150 bp paired-end reads (150PE) for a total of 100M reads after the removal of low-quality reads. Quality of raw data will be confirmed by FASTQC. Reads will be aligned to the latest version of the human reference genome (hg38) with BWA-MEM[112]. SNVs and Indels will be called using both MuTect2[113] and VarScan2[114] using tumor-normal pairs as input. Cross-comparison of calls between the tools will be used to collapse variants and reduce false-positives from germline variation.

CNV: DNA from residual lesions will be submitted for array genomic competitive hybridization (aCGH) using the Agilent SurePrint G3 4x180k array using corresponding germline DNA as reference. Quality of the array data will be assessed based on the standard QC metrics from Agilent's Feature Extraction software. Raw log ratios of copy numbers between matching tumor and buffy coat samples will be segmented using Circular Binary Segmentation in the ADaCGH2 Bioconductor package,[115] followed by the mergeLevels postprocessing step in the same package. Copy number gains and losses will be called by fixed thresholds determined based on the distribution of the segmented and merged copy number data.

13.2.3 DTC Assessment by Fluorescence-Activated Cell Sorting ("DTC-Flow")

For the DTC-Flow assay, a bone marrow aspirate sample drawn from the iliac crest under anesthesia will be collected in an anti-coagulated tube containing EDTA and stored at room temperature until DTC-Flow processing (within 48 hours of sample collection). Bone marrow aspirates will be processed for flow cytometric analysis using a stain-lyse-no wash protocol in which samples are first stained with

fluorescently-conjugated antibodies recognizing a panel of DTC markers, along with the hematopoietic cell marker CD45. Bone marrow aspirates will be incubated with magnetic particles conjugated to antibodies against CD45, enabling the depletion of CD45-positive bone marrow cells, and then subjected to red blood cell lysis and DAPI staining to distinguish live versus dead cells. DTCs will be detected by flow cytometry on a fluorescence-activated cell sorter (FACS). DTCs will be identified as DAPI-negative (live), CD45-negative (non-hematopoietic) cells that are positive for expression of one or more DTC markers, and sorted for downstream genomic analyses. Standardization of cytometry and sorting parameters will be performed using defined fluorescent particles, allowing the use of consistent sorting gates established through the analysis of multiple stained bone marrow aspirates collected from healthy donors.

A variety of methods have been developed for single cell genomic analysis, and this field is rapidly evolving. Below, we have prioritized technical approaches, however these choices would be modified and updated according to technical developments in the field[116-129].

DTC allocation to produce pooled biological replicates: To mitigate the effect of amplification on library complexity resulting from sampling drop-out, DTCs isolated from patients by DTC-Flow will be allocated to 9 different pools of DTCs (3 pools for RNA-seq; 3 pools for WES, and 3 pools for aCGH). Each pool will be amplified and sequenced separately. For patients with fewer than 9 DTCs, priority will be given to pools allocated for WES and aCGH.

RNA-seq: The three pools of DTCs allocated to RNA-seq will be sequenced using SMART-seq2 methodology[130], which enriches, amplifies, and processes polyadenylated transcripts into libraries for RNA-sequencing in one pipeline. SMART-Seq v4 Ultra Low Input RNA Kits are commercially available (Illumina) and are compatible with HiSeq 4000 sequencing. Resulting non-zero normalized read counts will be averaged across pools and used as features for gene expression analyses.

WES: DTC pools allocated to WES will be whole-genome amplified using multiple-displacement-amplification (MDA), which has been demonstrated to produce libraries with high coverage (>90%) across the targeted exome, with fewer false positives resulting from amplification errors than other methods[131]. Following amplification, libraries will be constructed using the SureSelect v5 Library Preparation kit. Libraries will be sequenced on the HiSeq 4000 with 150 bp paired-end reads (150PE). Only SNV calls that are present in at least two pools will be considered as true positives.

CNV: DTCs pools allocated to aCGH will be whole-genome amplified using multiple annealing- and looping-based amplification cycles (MALBAC). MALBAC produces a more uniform distribution of coverage across the genome, thus making it more suitable for CNV assays than amplification from MDA[132]. DNA will be hybridized to SurePrint G3 Human 4 x 180k aCGH arrays. Resulting signals will be segmented and compared among the three pools to identify true positive CNAs.

Residual tumor genomics: RNA-seq, WES and aCGH on residual tumor specimens will be performed as described in 2.3.1.

Circulating tumor genomics: Genomic analysis of CTCs and ptDNA will be performed as previously described.

13.2.4 ptDNA Analysis

2 10 ml blood samples will be obtained for ptDNA at each time point per the study calendar. Plasma will be processed within 2 hours of collection using our standard protocol and stored until ready for use. Preparation of plasma within 1 to 2 hours of venipuncture is a critical parameter for maintaining high quality plasma DNA [133]. Streck tubes (Cell-Free DNA BCT) for blood collection will be used, as we have recently published the ability of this tube to store blood samples at room temperature for up to 7 days without loss of plasma DNA integrity [134].

13.2.5 CTC analysis

Two 10 mL whole blood samples will be collected in EDTA Blood Collection Tubes at each time point per the study calendar. Buffy coat will be processed within 2 hours of collection using our standard protocol. Cytospin slides will be prepared with 1 million cells per slide for immunofluorescent staining. The remaining cells will be frozen as indicated in the standard processing protocol. . The markers used for CTC identification will include CD49c, CK, EpCAM, and HER2. CD45 will be used to distinguish WBCs (CD45 positive) from CTCs (CD45 negative).

13.3 Genetic Testing

Participants will be given information as part of the informed consent process that samples will be used for research tests that will include genetic studies and testing. The intent is not to give participants (or his/her medical providers) the results of any testing done for research purposes; however, incidental germline (heritable) mutations may be identified of which a participant may or may not already be aware. In the case that an incidental genetic finding is identified, the Protocol Chair of this project will be notified. The possible decisions for handling incidental findings may include notification of the participant (and provider); recommendation for genetic counseling, which may or may not include genetic testing (e.g., if the finding was not done in a CLIA certified laboratory); or, neither. In general, a member of the participant’s treating team will be given the information to help with notification. In all cases, the current policy of the University of Pennsylvania and local/participating site IRB, as applicable, will be followed and any additional approvals that may be required prior to participant notification will be secured in advance.

14 DATA HANDLING AND RECORD KEEPING

14.1 Confidentiality

Information about study patients will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability

Act (HIPAA). Those regulations require a signed patient authorization informing the patient of the following:

- What protected health information (PHI) will be collected from patients in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research patient to revoke their authorization for use of their PHI.

In the event that a patient revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of patient authorization. For patients that have revoked authorization to collect or use PHI, attempts should be made to obtain permission to collect at least vital status (i.e. that the patient is alive) at the end of their scheduled study period.

14.2 Source Documents

Source data is all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents. Examples of these original documents, and data records include: hospital records, clinical and office charts, laboratory notes, memoranda, evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, x-rays, and patient files.

14.3 Case Report Forms

The study case report form (CRF) is the primary data collection instrument for the study. All data requested on the CRF must be recorded. All entries will be entered into an electronic data capture system (EDC) via Oracle Clinical. Mandatory CRFs used for reporting to the ACC DSMC will be entered into Penn CTMS/VELOS.

15 STUDY FINANCES

15.1 Funding Source

This trial will be funded via the 2-PREVENT Breast Cancer Translational Center of Excellence and the NIH Research Project Grant: R01-CA208273. Novartis will provide commercial EVE to study participants. The DOD will support some of the correlative research using samples collected as part of this study.

15.2 Conflict of Interest

None to declare.

15.3 Patient Stipends or Payments

Patients will not be paid for participating in this clinical trial. However, assistance may be provided with parking and transportation and the costs of non-standard tests.

16 GENERAL REGULATORY CONSIDERATIONS AND CREDENTIALING

16.1 Publication of results

Any formal presentation or publication of data from this trial may be published after review and comment by Novartis and prior to any outside submission. Novartis

must receive copies of any intended communication in advance of publication (at least fifteen working days for presentational materials and abstracts and thirty working days for manuscripts). These requirements acknowledge Novartis' responsibility to provide peer input regarding the scientific content and conclusions of such publications or presentations. Principal Investigation/Institution shall have the final authority to determine the scope and content of its publications, provided such authority shall be exercised with reasonable regard for the interests of Novartis and, in accord with the trial contract and shall not permit disclosure of Novartis confidential or proprietary information.

16.2 Disclosure and confidentiality

The investigator agrees to keep all information provided by Novartis in strict confidence and to request similar confidentiality from his/her staff and the IRB/IEC/REB. Study documents provided by Novartis (investigators' brochures and other material) will be stored appropriately to ensure their confidentiality. The information provided by Novartis to the investigator may not be disclosed to others without direct written authorization from Novartis, except to the extent necessary to obtain informed consent from patients who wish to participate in the trial.

16.3 Ethics and Good Clinical Practice

This study must be carried out in compliance with the protocol and the principles of Good Clinical Practice, as described in Novartis standard operating procedures and:

- ICH Harmonized Tripartite Guidelines for Good Clinical Practice 1996. Directive 91/507/EEC, The Rules Governing Medicinal Products in the European Community.
- US 21 Code of Federal Regulations dealing with clinical studies (including parts 50 and 56 concerning informed consent and IRB regulations). Declaration of Helsinki and amendments, concerning medical research in humans (Recommendations Guiding Physicians in Biomedical Research Involving Human Subjects).

The investigator agrees to adhere to the instructions and procedures described in it and thereby to adhere to the principles of Good Clinical Practice that it conforms to.

16.4 Institutional Review Board/Independent Ethics Committee

Before implementing this study, the protocol, the proposed informed consent form and other information to subjects, must be reviewed by a properly constituted Institutional Review Board/Independent Ethics Committee/Research Ethics Board (IRB/IEC/REB). A signed and dated statement that the protocol and informed consent have been approved by the IRB/IEC/REB must be given to Novartis before study initiation. Any amendments to the protocol, other than administrative ones, must be reviewed by Novartis approved by this committee.

16.5 Informed consent

The investigator must explain to each subject (or legally authorized representative) the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved and any discomfort it may entail. Each subject must be informed that participation in the study is voluntary and that he/she may withdraw from the study at any time and that withdrawal of

consent will not affect his/her subsequent medical treatment or relationship with the treating physician.

This informed consent should be given by means of a standard written statement, written in non-technical language. The subject should read and consider the statement before signing and dating it, and should be given a copy of the signed document. If the subject cannot read or sign the documents, oral presentation may be made or signature given by the subject's legally appointed representative, if witnessed by a person not involved in the study, mentioning that the patient could not read or sign the documents. No patient can enter the study before his/her informed consent has been obtained.

The informed consent form is considered to be part of the protocol, and must be submitted by the investigator with it for IRB/IEC/REB approval.

16.6 Declaration of Helsinki

The investigator must conduct the trial in accordance with the principles of the Declaration of Helsinki. Copies of the Declaration of Helsinki and amendments will be provided upon request or can be accessed via the website of the World Medical Association at http://www.wma.net/e/policy/17-c_e.html.

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18 Appendices

Informed consent form