Title: Chemotherapy Before Surgery and Tissue Sample Collection in Patients with Stage IIA-IIIC Breast Cancer

IRB ID: **13-02-079**
NCT01897441
IRB Approval Date: **05/11/2021**

Version 4.2 26 April 2021

Title: Prospective tissue collection in breast cancer patients receiving preoperative systemic therapy

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Summary of Protocol Changes :

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- Amendement 1 (Version 4.1 1/9/17) : (1) Cover page: removed Dr. Montgomery, Koenigsberg, McDaid and co-investigators; (2) Schema and section 4.1: eliminate pre-randomization, all patients receive sequential pacitaxel-AC (unless physician chooses AC-paclitaxel sequence), (3) Schema and section 4: trastuzumab (+/- pertuzumab) plus carboplatin/docetaxel or paclitaxel added as a treatment option for HER2-positive disease to reflect a new standard of care option, additional stratum added (stratum III) to include neaodjuvant endocrine therapy (4) Schema and section 5.1 : prior version allowed FNA or FNA followed by core biopsy, which was more made more explicitly clear in current protocol version and consent, and reduced optional core biopsies from 5 to 3, (5) consolidated background section and study objectives, (6) statistical section and accrual goal modified, (6) deleted Appendix A (RCB staging) and B (AJCC staging), as these are considered part of standard care

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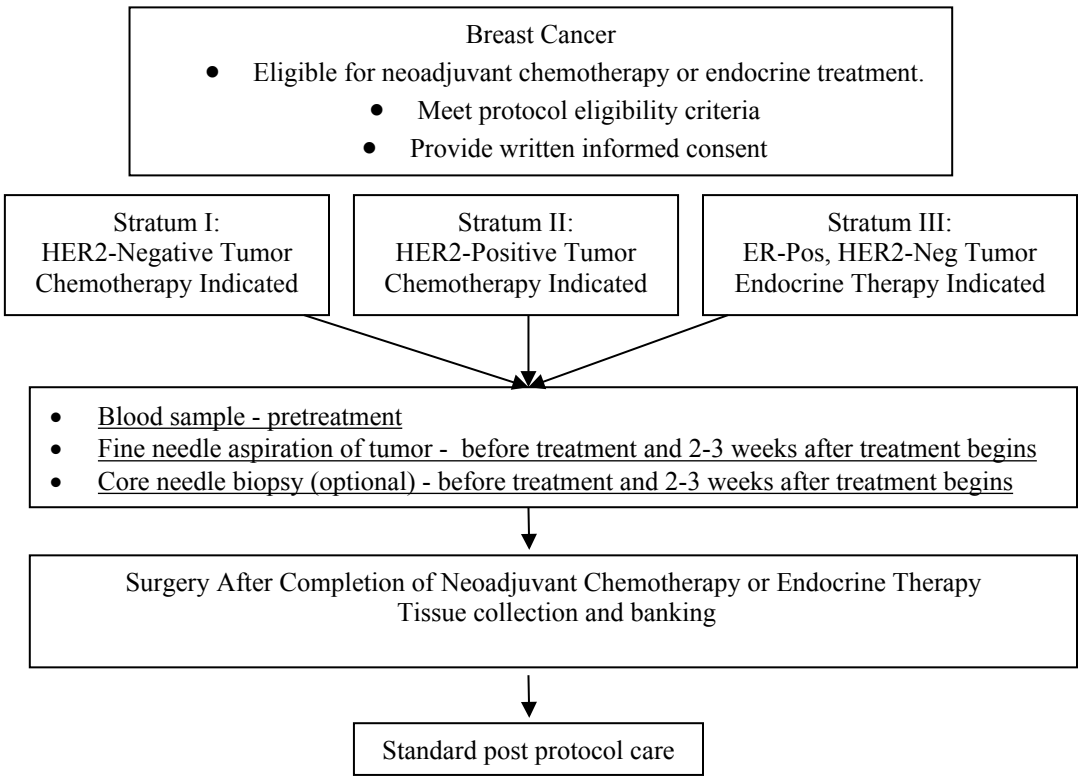
SCHEMA


Breast Cancer
Eligible for neoadjuvant chemotherapy or endocrine treatment.
Meet protocol eligibility criteria
Provide written informed consent
Stratum I: HER2-Negative Tumor Chemotherapy Indicated
Stratum II: HER2-Positive Tumor Chemotherapy Indicated
Stratum III: ER-Pos, HER2-Neg Tumor Endocrine Therapy Indicated
Blood sample - pretreatment
Fine needle aspiration of tumor - before treatment and 2-3 weeks after treatment begins
Core needle biopsy (optional) - before treatment and 2-3 weeks after treatment begins
Surgery After Completion of Neoadjuvant Chemotherapy or Endocrine Therapy
Tissue collection and banking
Standard post protocol care

1. HYPOTHESIS & OBJECTIVES

We hypothesize that the chemotherapy combinations/agents that are commonly used to treat breast cancer (including doxorubicin/cyclophosphamide, paclitaxel trastuzumab, pertuzumab, carboplatin/docetaxel) will have different biological outcomes on breast cancer cell fate; specifically cell death versus senescence. Accelerated senescence is a potent tumor suppressive response that halts the proliferation of cancer cells following treatment with cytotoxic chemotherapy. Our hypothesis predicts that **persistent** senescent cells and or / senescent reverted cells drive disease progression, and/or epithelial to mesenchymal transition (EMT). This is mediated via the pro-inflammatory activity of the senescence-associated secretory phenotype (SASP), a unique property of senescent cells and their resistant counterparts. Thus, the presence of these cells within a tumor mass likely contributes to disease progression, drug resistance and metastasis, ultimately leading to death.

We propose to evaluate the effects of commonly used standard chemotherapy regimens on the propensity for senescence, and as a consequence EMT; and to create a biorepository for future studies.

Objectives:

(1) To evaluate the effects of preoperative neoadjuvant chemotherapy (+/- anti-HER2 therapy) on invasive Mena isoforms (ie $Mena^{inv}$, $Mena^{Calc}$), TMEM score, and cancer stem cells
(2) To create a biospecimen repository for future studies derived from patients with breast cancer receiving standard neoadjuvant chemotherapy.

2. BACKGROUND

a) Preoperative Neoadjuvant Chemotherapy

A metanalysis of 9 randomized trials including 3496 patients with operable breast cancer has shown comparable survival rates whether chemotherapy was given preoperatively (neoadjuvant) or postoperatively (adjuvant)[1]. Patients with operable breast cancer typically undergo surgery first followed by adjuvant chemotherapy, but neoadjuvant chemotherapy may be indicated in certain clinical scenarios, including: (1) locally advanced inoperable breast cancer (eg, inflammatory disease, large primary tumor, bulky adenopathy) in which breast conservation is not an option without therapeutic downstaging induced by systemic therapy, (2) operable disease in which downstaging with systemic therapy may facilitate beast conservation when it would otherwise might not have been feasible.[2-4] ***The goal of neoadjuvant chemotherapy is to induce a pathologic complete response (pCR) in the breast or breast and lymph nodes, which has been shown to be a short-term surrogate indicative of long-term cure.*** [2-4] In fact, the U.S. Food and Drug Administration has recently issued draft guidance recognized the value of pCR in the breast and lymph nodes after neoadjuvant chemotherapy as an acceptable endpoint in clinical trials supporting accelerated drug approval of a new agent/regimen. [5]

b) Type and Sequence of Preoperative Chemotherapy Regimens

As previously stated, the goal of neoadjuvant chemotherapy is to induce a pCR. Approximately 10% of patients with operable breast cancer achieve breast pCR after 4 cycles of neoadjuvant AC (doxorubicin 60 mg/m2, cyclophosphamide 600 mg/m2) given every 3 weeks.[6] A subsequent trial (B27) that compared preoperative sequential AC x 4 followed by docetaxel (100 mg/m2 every 3 weeks x 4) to AC alone x 4 demonstrated a significant improvement in pCR from 14% to 26%. [7] Other studies have shown that weekly paclitaxel therapy (80 mg/m2 weekly x 12) to be associated with a higher pCR rate in the breast and lymph nodes compared with paclitaxel given every 3 weeks (225 mg/m2 x 4) sequentially with an anthracycline-cyclophosphamide combination [8], including all patients treated (28.2% 15.7%; P = .02), and patients with both ER-negative disease (48% vs. 23%) and ER-positive disease (22% vs. 11%).

The addition of HER2-directed therapy (ie, trastuzumab) to chemotherapy has been shown to further improve pCR rate.[9] ***Sequential AC-taxane therapy (plus trastuzumab for HER2-positive disease) has therefore become an accepted standard of care for patients who are candidates for both postoperative adjuvant chemotherapy and preoperative neoadjuvant chemotherapy.***

Evidence suggests that the paclitaxel-AC sequence of drug administration may be more effective than the standard sequence of AC-taxane therapy. Antiangiogenic therapy improves blood flow in tumors that exhibit disordered vascular beds, thereby improving drug delivery of cytotoxic agents.[10] Taxanes have potent angiogenic effects[11], and may also improve tumor blood flow by reducing tumor-associated vascular compression.[12] Preoperative paclitaxel more effectively reduced interstitial fluid pressure (IFP) in primary breast cancers than AC in a study which included 54 patients with breast cancer treated with neoadjuvant AC or weekly paclitaxel.[13] Weekly paclitaxel, when administered first, decreased the mean IFP by 36% (P = .02) and improved the tumor pO(2) by almost 100% (P = .003). In contrast, doxorubicin did not have a significant effect as measured by ultrasound. These changes were <u>independent</u> of the tumor response to both drugs as evaluated by ultrasound, and the study was not powered to demonstrate a significant difference in clinical outcome based on the sequencing of drugs. Recent studies in mouse models suggested that doxorubicin downregulates the surface programmed cell death 1 ligand 1 (PD-L1) in certain breast cancer cell types, which is a co-inhibitory molecule for cytotoxic T cells. Thus, it is hypothesized that this that might at least partially increased immunogenic apoptotic death.[14] In contrast, in MDA-MB-468 breast cancer cell culture models, paclitaxel promotes PD-L1 mediated T cell apoptosis. These findings reveal a potential link between taxane chemotherapy and immunoresistance.[15] Although it remains unknown whether the efficacy of anthracycline and paclitaxel combinations is affected by their sequence of administration, one phase III trial demonstrated a significantly higher pCR rate when the taxane followed by an anthracycline/cyclophosphamide-containing regimen was compared with the reverse sequence (20% vs. 15%, p=0.03), suggesting that the reverse sequence (taxane ⇒AC) may be somewhat more effective than the standard AC ⇒ taxane sequence. [8] In addition, a neoadjuvant randomized phase III trial (Neo-tAnGo) in women with high-risk early breast cancer demonstrated a significant advantage in pCR (20% vs15%) with the sequence of paclitaxel+/-gemcitabine compared with the more conventional anthracycline first sequencing.[16] Of note, the benefit was higher for the ER positive group. Moreover, a recent restrospective analysis of the clinical outcome of the two sequences in a large number of patients treated with neoadjuvant therapy reported that the sequence of paclitaxel-anthracycline compared to the antracycline-paclitaxel is associated with lower risk of relapse.[17] ***Therefore, although either sequence of drug administration, (AC ⇒ taxane sequence, or taxane⇒ AC sequence), are considered acceptable regimens for neoadjuvant chemotherapy treatment, there is tentative evidence that supports superior 'biological' and clinical activity for taxane⇒ AC regimen.***

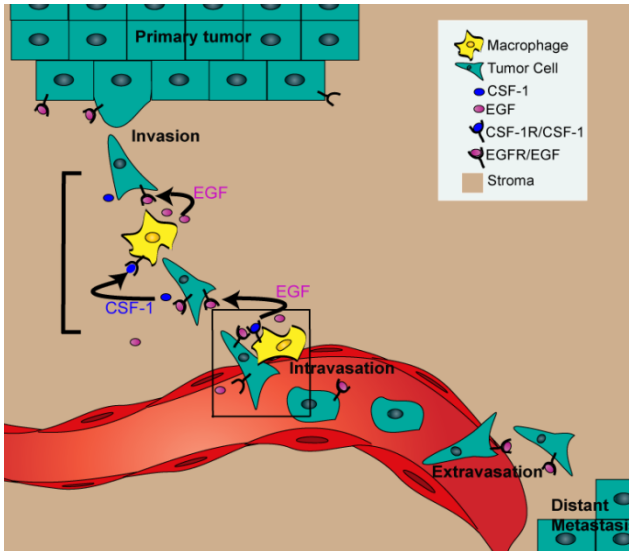


Figure 1: Migration of discohesive tumor cells leads to intravasation in mammary tumors. *Tumor cell migration is believed to involve the tumor microenvironment-initiated decreased expression of the epithelial isoform of Mena, Mena11a, and increased expression of Mena^{INV} which promotes discohesion of the tumor and paracrine-dependent tumor cell migration (bracket), as well as TMEM assembly leading to intravasation (box).*

<u>c) TMEM as a measure of Cancer Cell Invasion and Interaction with Microenvironment</u>

Highly invasive and motile cells are capable of metastasizing, non-proliferating and chemotherapy resistant. A multidisciplinary team of scientists from the Condeelis laboratory at Einstein and clinicians at

Montefiore have shown that only a subset of tumor cells is capable of metastasizing and that these cells show reduced response to chemotherapy and express a distinct set of genes and their splice variants.[18] Using multiphoton-based intra-vital imaging in mouse and rat mammary tumors the team identified a subpopulation of cancer cells that is discohesive and migratory (shown in bracket in **Figure 1**) and demonstrated that these discohesive, migratory tumor cells intravasate when associated with peri-vascular macrophages. [19-22]. In addition, an *in vivo* invasion assay developed by Condeelis and Goswami has facilitated collection of invasive tumor cells from mammary tumors of rats, mice, and humans for expression profiling [23,24]Many of the epigenetic changes in these cells are clustered in the motility pathways that control actin polymerization, directional cell movement toward EGF, and invadopodium formation [25,26]. The Mena family of actin binding proteins functions at the convergence of these motility pathways ***implying that Mena expression levels reflects the activity of motility pathways involved in the migratory/metastatic potential of cancer cells.*** Thus, Mena expression level is used as a summary marker for the motility pathways [24,26-28]. Mena promotes actin polymerization by enhancing the activation of cofilin via increasing the sensitivity of the EGFR to EGF, interfering with the activity of inhibitory capping proteins and increasing actin filament elongation rates [26,29]. This activity is essential for sustained directional cell movement in response to growth factors like EGF. Mena knockout mice crossbred with PyMT-oncogene carrying mice develop dramatically less metastatic mammary tumors [30]. Furthermore, the survival of these animals was prolonged due to the almost complete absence of hematogenous dissemination of tumor cells from the primary tumor [30]. Breast carcinoma cells express three Mena isoforms: Mena^{classic}, Mena^{INV} and Mena11a. Mena^{classic} contains only the constitutive exons, while the two splice variants Mena^{INV} or Mena 11a contain the alternately-included exons INV or 11a, respectively. Mena is up regulated in rat, mouse and human mammary tumors [19]. The Condeelis laboratory discovered that the observed increase of Mena expression in invasive and disseminating tumor cells reflects increased expression of both Mena^{classic} and Mena^{INV}, and correlates with decreased Mena 11a expression relative to non-invasive, non-intravasating tumor cells within the primary mammary tumor [19]. They demonstrated that expression of Mena^{INV} helps increase invasiveness of tumor cells *in vitro* and *in vivo*, and metastatic activity in mammary tumors *in vivo* by increasing the sensitivity of the EGFR to EGF by 50 fold which increases paracrine signaling between macrophages and tumor cells (Fig. 1, bracket) [29,31]. It is believed that Mena^{classic} co-oligomerizes with Mena^{INV} to form a Mena^{classic}/Mena^{INV} hetero-tetramer so that they are co-localized and function together [19,26,32]. On the other hand, Mena 11a is associated with epithelial growth [33] and epithelial integrity [34] in breast tumors. Thus cancer cells overexpressing Mena^{classic}/Mena^{INV} hetero-tetramer may be essential for intravasation step of metastasis.

Dr. Oktay has identified the discohesive migratory, Mena^{INV} expressing cell population shown in the bracket in **Figure 1** in fine needle aspirate (FNA) samples from human invasive ductal carcinomas of the breast and shown that the level of Mena^{INV} expression correlates with the number of macrophage assisted cancer cell intravasation sites called TMEM (***"tumor microenvironment of metastasis"***, box in **Figure 1**) [35]. As mentioned above, TMEM intravsation sites have been identified in mouse by intravital imaging. They have been also identified in humans by triple immunostain using anti CD-31 antibodies for endothelial cells, anti-CD68 for macrophages and anti-Mena for cancer cells. Furthermore, TMEM score has been shown to correlate with the development of distant metastases in humans independent of other clinicopathologic features [36]. ***Interestingly, the induction of cell death by cytotoxic agents such as doxorubicin is much lower in invasive migratory cell population than in the cells isolated from the whole tumor profiling [23,24], suggesting that Mena isoform expression and TMEM status can identify patients who are not good candidate for chemotherapy because they have high proportion of invasive, metastatic chemoresistant cells. Immunohistochemical (TMEM score) and cytological (Mena isoform expression) assays developed by Drs. Condeelis, Oktay and Goswami can be easily incorporated into current clinical practice and used to identify patients with high risk of metastasis.***

d) Rationale for amdendment 1 (1/9/17): The protocol was modified to eliminate prerandomization for drug sequence (paclitaxel – AC vs. AC – paclitaxel) for patients with HER2-negative disease, as paclitaxel –AC sequence is an accepted standard, and to reflect changing standard of care for HER2-positive disease (TCH-P). Based on preliminary data generated from the first 14 patients enrolled indicating that chemotherapy induces expression of invasive Mena isoforms (comparing pre-treatment FNA/biopsy with during treatment FNA/biopsy) and TMEM score (comparing pre-treatment biopsy and

post-treatment residual cancer), we now have focused the aims of this protocol on obtaining additional information regarding the effect of chemotherapy on invasive Mena isoforms, TMEM score, and cancer stem cells, and to clarify that patients who agree to only FNA (but decline core biopsy) are eligible.

3. PATIENT SELECTION

3.1 Eligibility Criteria

3.1.1 Patients must have histologically confirmed adenocarcinoma of the breast associated with the following clinical stage: IIA, IIB, IIIA, IIIB, or IIIC (see AJCC staging criteria, 7h Edition). Patients with stage IV disease are also eligible if there is an intention to perform breast surgery after neoadjuvant therapy is completed, or in patients participating in clinical trials where surgery after neoadjuvant therapy may be an option.

3.1.2 ER, PR, and HER2/neu status documented by core needle biopsy of the primary tumor and/or regional lymph node must be known prior to beginning systemic therapy.

3.1.3 Patients must have had a bilateral diagnostic mammogram within 6 months of registration, and may also have a targeted sonography of the breast and/or ipsilateral axilla and MRI if clinically indicated.

3.1.4 Patients with clinically suspicious axillary lymph node involvement must have either aspiration cytology or biopsy prior to beginning therapy.

3.1.5 It is strongly encouraged that all patients have metallic clips placed in the tumor prior to neoadjuvant therapy in order to facilitate evaluation for microscopic disease at the time of surgery; placement of clips is particularly encouraged for patients being considered for breast conserving surgery.

3.1.6 No prior chemotherapy, irradiation, or definitive therapeutic surgery (eg, mastectomy or lumpectomy or axillary dissection) for this malignancy. Patients who have had a prior sentinel lymph node biopsy for this malignancy are eligible.

3.1.7 Patients who received tamoxifen or another selective estrogen receptor modulator (SERM) for prevention or for other indications (e.g., osteoporosis, prior DCIS) are eligible. Tamoxifen therapy or other SERMs should be discontinued at least 1 week before the patient is enrolled on this study.

3.1.8 Age ≥18 years. Because breast carcinoma is a disease of adults that rarely occurs in children, children are excluded from this study.

3.1.9 The patient is medically suitable candidate for preoperative chemotherapy and surgery in the judgment of the treating physicians.

3.1.10 Ability to understand and the willingness to sign a written informed consent document, and to willing to provide blood samples before and during preoperative therapy. Patients are also asked but not required to have research biopsies performed before and after therapy.

4. MANAGEMENT PLAN

4.1 Treatment

Note:..All drugs doses are based on actual body weight, and are regarded as standard of care. See section 5.2 for criteria for dose modification/delay.

4.1.1 Paclitaxel alone (Stratum I) or plus Trastuzumab +/- Pertuzumab (Stratum II)

- **Paclitaxel (Stratum I or II):** 80 mg/m2 IV infusion over 1 hour weekly x 12 consecutive weeks. Paclitaxel premedication: administer dexamethasone 10 mg IV prior to the first paclitaxel dose. If no reaction after the first dose administer dexamethasone 4 mg IV (or lower doses if well tolerated without reaction), diphenhydramine 25-50 mg IV, and an H2 blocker (cimetidine 300 mg, ranitidine 20 mg, or an equivalent) approximately 30-60 minutes prior to each paclitaxel dose. Proceed with each weekly dose of paclitaxel (+/-

trastuzumab +/- pertuzumab) if:

- Absolute neutrophil count ≥ 1000/uL and platelets ≥ 50,000/uL
- No grade 3-4 toxicity, or prohibitive grade 2 toxicity
- No evidence of clinical cardiac dysfunction

- **Trastuzumab (+/- pertuzumab) plus paclitaxel (Stratum II- HER2-positive disease only):** Trastuzumab 8 mg/kg IV loading dose over 90 minutes, then 6 mg/kg every 3 weeks (after paclitaxel when given concurrently) for a total of 17 doses (51 weeks) To be administered after paclitaxel. Pertuzumab (840 mg IV loading dose cycle 1, then 420 mg every 3 weeks for 6 cycles prior to surgery) may also be used at the discretion of the treating physician.
- **TCH+P or TCH - docetaxel/carboplatin, trastuzumab +/- pertuzumab (Stratum II:** Docetaxel 75 mg/m2 and carboplatin AUC 6 (Calvert formula) every 3 weeks for 6 cycles, plus ,trastuzumab (8 mg/kg loading dose cycle 1, then 6 mg/kg every 3 weeks) for up to 17 doses before and after surgery (51 weeks), and pertuzumab (840 mg IV loading dose cycle 1, then 420 mg every 3 weeks for 6 cycles prior to surgery). Patients to receive standard antiemetic premedication, plus dexamethasone 8 mg PO BID x 3 days beginning 1 day prior to each cycle. Pertuzumab may be omitted at discretion of treating physician. Repeat every 3 weeks if:
  - Absolute neutrophil count ≥ 1000/uL and platelets ≥ 50,000/uL
  - No grade 3-4 toxicity, or prohibitive grade 2 toxicity
  - No evidence of clinical cardiac dysfunction
- **Granulocyte colony stimulating factor (G-CSF; filgrastim):** G-CSF should not be used during paclitaxel unless: (1) treatment with paclitaxel is delayed due to neutropenia, (2) the patient has a serious or life-threatening documented or suspected infection associated with neutropenia (absolute neutrophil count [ANC]< 1000/mm3), or (3) or another complication that in the judgment of the treating physician could derive potential benefit from G-CSF. The drug should be used at a dose/schedule specified in the package insert. Pegfilgrastim should not be used during paclitaxel therapy. Pegfilgrastim (6 mg) should be given after each AC dose if given on an every 2 week schedule, or after TCH+/-P at discretion of treating physicians.

4.1.2 Doxorubicin and Cyclophosphamide (AC) – Stratum I or II

- Doxorubicin 60 mg/m2 IV over 5-10 minutes, Cyclophosphamide 600 mg/m2 IV infusion over 30-60 minutes. To be given 3very 2 weeks x 4 cycles. When given after paclitaxel, the first cycle should be initiated 3 weeks after the last paclitaxel and trastuzumab dose, or 2 weeks after the last paclitaxel dose when used without trastuzumab. For patients in stratum II, AC may be omitted at discretion of treating physician depending upon clinical response to therapy, toxicity, or other factors, in accordance with standard clinical practice.
- Premedication: Administer antiemetic therapy in accordance with NCCI guidelines.
- Pegfilgrastim 6 mg SC on day 2 of each cycle
- Repeat every 2 weeks x 4 cycles if: (a) absolute neutrophil count ≥1500/uL, platelets ≥100,000/uL, (b) recovered from non-hematologic toxicity to grade 1 or less (except alopecia)

4.13 Neoadjuvant Endocrine Therapy – Stratum III

Patients with ER-positive, HER2-negative disease may receive neoadjuvant endocrine therapy with an aromatase inhibitor (anastrozole 1 mg po daily, letrozole 2.5 mg daily, or exemestane 25 mg po daily) for 4-6 months prior to surgery (or longer if clinically indicated).

4.1.4 Chemotherapy Dose Modifications (StrataI & II)

- Proceed with each dose of chemotherapy if:
  - Absolute neutrophil count ≥ 1000/uL and platelets ≥ 50,000/uL (> 100,000/uL for AC)

- No persistent grade 3-4 toxicity, or prohibitive grade 2 toxicity
- No evidence of clinical cardiac dysfunction

- Patients who experience the toxicities outlined below should have the chemotherapy dose modified according to the criteria outlined in the table below.

Toxicity	**Paclitaxel or AC**
Grade 3 or 4 febrile neutropenia (fever ≥ 38.5°C and ANC < 1,000/mm3):	Reduce 1 dose level
Grade 3 or 4 non-hematologic toxicity	Reduce 1 dose level
Grade 2 non-hematologic toxicity	Reduce 1 dose level if poorly tolerated and attributable to therapy

Dose Reduction	**Paclitaxel Dose**	**AC Dose**	**Docetaxel Dose**	**Carboplatin Dose**
Initial Dose	80 mg /m2	A-60 mg/m2; C-600 mg/m2	75 mg/m2	AUC6
First Reduction	Reduce 25% - 60 mg/m2	Reduce 25% (A45, C450 mg/m2)	60 mg/m2	AUC4.5
Second Reduction	Reduce 50% - 45 mg/m2	Reduce 50% (A30, C300 mg/m2)	45 mg/m2	AUC3

4.1.5 Surgery

Patients will be reassessed for surgery following completion of chemotherapy or endocrine therapy. Primary surgery may consist of a modified radical mastectomy, radical mastectomy, segmental mastectomy or lumpectomy with an axillary lymph node dissection, or sentinel lymph node biopsy without further axillary dissection if the sentinel node biopsy is negative if deemed medically appropriate. The goal of surgery is to completely debulk the tumor and achieve tumor free margins. The local pathologist should carefully evaluate the specimen for microscopic residual disease, including searching for the prior biopsy tract and/or metallic clips placed preoperatively. The surgery should be performed about 4 weeks (range of 2-6 weeks) after the last chemotherapy dose unless it needs to be delayed because of persistent adverse events or other medical problems.

4.1.5 Post-Protocol Therapy

The following portions of the treatment are recommended after surgery in accordance with the standard of care:

- Adjuvant chest wall/breast irradiation if standard indications met (eg, if tumor > 5 cm, inflammatory carcinoma, 4 or more positive axillary nodes, or 1-3 positive nodes with extranodal extension or large metastasis)
- Adjuvant hormonal therapy for a minimum of 5 years (for patients with ER and/or PR-positive disease)
- Postoperative trastuzumab (for up to 51 weeks, or up to 17 doses of trastuzumab given before and after surgery (if HER2-positive).

5. SPECIMEN COLLECTION AND BIOPSY PROCEDURES

5.1. Tumor Fine Needle Aspiration +/- Core Biopsies

Consented patients will undergo serial tumor sampling and blood specimen collection at:

- Baseline (before treatment initiation)
- 2-3 weeks after the first chemotherapy dose
  - Stratum I: 2 weeks after the first chemotherapy dose, (ie, just before the 3rd planned paclitaxel dose)
  - Stratum II: 2 weeks after first chemotherapy dose (ie, just before the 3rd planned paclitaxel dose if paclitaxel dose) or 3 weeks after first chemotherapy dose (if carboplatin/docetaxel used
- At the time of definitive surgery (residual tumor/ tumor bed - section 7.0).

Biospecimens will be collected and the study conducted in accordance with the recommendations of the NCI-BIG Breast Cancer Intergroup[37,38].

Procedures for collection and processing specimens will be stipulated in detail in a Standard Operation Procedure Manual developed specifically for this protocol. A recent study evaluated the aliquots of fresh primary tumor tissue from 17 surgically resected invasive breast cancers were placed into RNA later at room temperature after tumor removal (baseline) and up to 3 hours thereafter or were snap frozen at baseline and 40 minutes thereafter.[39] Although sample preservation in RNAlater was associated with improved RNA yield and quality, whereas cold ischemia increased RNA fragmentation as measured by the 3'/5' expression ratio of control genes, expression levels of single genes and multigene signatures that are of diagnostic relevance in breast cancer were mostly unaffected by sample preservation method, or prolonged cold ischemic duration. This indicates that for

The following guidelines apply to sampling and specimen processing:

- All patients will have fine needle biopsy (FNA) procedure), sonographically guided if necessary. Patients may elect to have: (1) FNA only, (2) FNA plus core biopsies
- The procedure may be billed to the insurance company if performed to confirm/establish a diagnosis or biomarkers (e.g., ER, PR, HER2) at baseline, but **not** in other circumstances (e.g. during treatment biopsy).
- Five FNA passes (all patients) followed 3 core biopsies (if patient agrees) will be obtained with 25 and 12 gauge needles respectively. The specimens should be processed a follows:
  - FNA: immediately placed in cytology solution
  - 1st core biopsy (if done): 10% formalin (to confirm histologic presence of tumor)
  - 2rd core biopsy (if done): RNA Later (for gene expression studies in future). RNAlater® samples should be shipped on a cold pack. For more information on RNAlater, see http://www.ambion.com/techlib/tn/114/6.html
  - 3rd : Snap frozen

5.2 Blood Specimens

- Draw peripheral blood using vacutainer into two Acid Citrate Dextrose (ACD tubes (yellow top, draw volume 6 ml/tube), then gently invert tube 8-10 times at baseline (before treatment).
- Label tube with patient ID number
- Place specimen on cool pack deliver specimen and form to Einstein Biorepository on same day.
- Samples will be processed in the Einstein biorepository for storage of peripheral blood mononuclear cells (PBMC's) and plasma.

6. CORRELATIVE STUDIES

6.1 TMEM

TMEM stain is a triple immunostain for predicting metastatic risk in which 3 antibodies are applied sequentially, and developed separately with different chromogens on a Bond Max Autostainer as described previously [36]. TMEM is defined as the direct apposition of an endothelial cell, a perivascular macrophage, and a Mena expressing tumor cell. The antibodies used are CD31 (endothelial cell), CD68 (macrophage) and pan Mena (intravasation competent tumor cell) [36]. A pan-Mena mouse monoclonal (A351F7D9) will be used to identify Mena positive tumor cells. The assessment of TMEM scores will be performed using Adobe Photoshop on 10 contiguous 400x digital images of the most representative areas of the tumor. The total TMEM for each image will be tabulated, and the scores from all ten images will be summed to give a final TMEM density for each patient sample, expressed as the number of TMEM per 10 400x fields (total magnification). Two pathologists will score TMEM, including Dr. Oktay. TMEM score will be obtained on pre-treatment core biopsies and the post-treatment excision.

6.2 qRT-PCR for Mena isoform expression and Flow Cytometry for Cancer Stem Cells

PCR analyses will be performed using SyBR Green kit and ABI 7300 sequence detector. PCR primers used for the detection of $Mena^{classic}$, $Mena^{INV}$ and Mena 11a isoforms have been described by us previously [19]. These primers do not recognize other Ena/VASP family members. Also, macrophages do not express Mena [40]. Therefore, PCR detects Mena levels in tumor cells only. PCR analysis will be performed on FNA samples obtained before treatment and on FNA samples obtained from surgical resections of chemoresistant tumors. The percentage of $CD44^{high}/CD24^{low}$ breast cancer stem cells (BCSCs) by flow cytometry will be quantified.

7. STUDY CALENDAR

Parameter	**Specimen Collection Time Points**	**Correlative studies**
Research Blood Samples	○ Pretreatment	• Biospecimen banking
Research Tumor Samples	○ Pretreatment ○ 2-3 weeks after first chemotherapy dose (section 5.1)	• TMEM (from pretreatment diagnostic core biopsy, and week 2-3 biopsy if done) • qPCR Mena isoform (FNA) • Cancer stem cells (FNA)
Tissue Collection at Surgery	○ At time of surgical procedure	• AJCC Y stage (routine pathlogy) • RCB Score (study pathologist) • TMEM, qPCR Mena isoform, and cancer stem cells in residual tumor if present

8. MEASUREMENT OF EFFECT

Pathological Response

The pathologic response to treatment will be assessed by in all patients as follows:

- "y staging" denoting prior neoadjuvant chemotherapy using AJCC Y version 7.0, as described in the pathology synoptic report
- Residual Cancer Burden" will be assessed by one of the study pathologists as described (http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3). [41]

9. REGULATORY AND REPORTING REQUIREMENTS

9.1 Registration and Data Reporting

- All patients must be screened for eligibility and meet all eligibility criteria before being offered participation in the trial
- Patients who meet eligibility criteria for stratum A may be consented for participation if the eligibility criteria are met.
- Patients who meet the eligibility criteria for stratum B or C (HER2-negative disease, candidate for preoperative chemotherapy) must be pre-randomized first with the Cancer Clinical Trials Office and receive a stratum assignment to stratum B or C. After pre-randomization, patients must be offered the trial and consented.
- Data and specimen collection will be collected using study specific case report form.

9.2 Adverse Event Reporting

- Adverse events will be reported in accordance with guidelines established by the Institutional Review Board (.http://www.einstein.yu.edu/docs/administration/institutional-review-board/policies/AE-internal.pdf). ***All of the treatment given as a component of this protocol is consistent with standard of care. Only the biopsies are considered research-related procedures and require adverse event reporting, including:***
  - **DEATH:** The following must be reported: (a) deaths that occur while subject is on study treatment, or (b) deaths that occur within thirty 30 days of conclusion of study intervention. **All deaths, whether or not anticipated or related to the protocol, must be reported to the designated IRB within 48 hours of the PI's knowledge of the death. Within 10 business days of informing the designated IRB, the Principal Investigator is required to submit a completed Adverse Event Report to the designated IRB.**
  - **SERIOUS EVENT:** A serious event is defined as (a) an adverse event, whether or not anticipated, that has the potential to cause significant impairment of health or death; or (b) an adverse event that prompts the Principal Investigator to suspend the research protocol, (temporarily or permanently) even if the risk is explicitly identified in the Consent Form; or (c) events that occur with a frequency or degree of severity greater than anticipated. (Examples of Serious Events are: disability, inpatient hospitalization, prolongation of hospitalization, congenital anomaly, laboratory anomalies critical to safety evaluations, and serious adverse drug reactions.) The following must be reported: (a) serious events that occur while subject is on study treatment, or (b) serious events that occur within thirty (30) days of conclusion of study intervention **All reportable serious events must be reported to the designated IRB within 48 hours by phone, e-mail, or fax. Within 10 business days of initial notification to the designated IRB the Principal Investigator is required to submit a completed Adverse Event Report.**
  - **NON-SERIOUS UNANTICIPATED EVENT:** A reportable non-serious unanticipated event is an adverse event that is not explicitly identified in the consent form and/or in the investigator's brochure, does not pose a serious impairment to a subject's health, and is not life-threatening. **Non-Serious Unanticipated Events must be reported to the designated IRB within 30 days via submission of a completed *Adverse Event Report.***

10. DATA SAFETY AND MONITORING BOARDS

All trials initiated by the Montefiore Medical Center are subject to oversight by the Albert Einstein Cancer Center Data Safety Monitoring Board (DSMB). This board meets once a month with any additional meetings scheduled when needed. The responsibilities are as follows:

- Familiarize themselves with the research protocol (s)
- Review interim analyses of outcome data and cumulative toxicity data summaries to determine whether the trial should continue as originally designed, should be changed, or should be terminated based on these data.
- The Albert Einstein Cancer Center DSMB reviews trial performance information such as accrual information.
- The Albert Einstein Cancer Center DSMB also determines whether and to whom outcome results should be released prior to the reporting of study results.
- All adverse events are reviewed by the committee with assurances that these have been in fact sent for review to all pertinent IRBs.
- Review of reports of related studies to determine whether the monitored study needs to be changed or terminated.
- Review major proposed modifications to the study prior to their implementation (e.g., termination, dropping an arm based on toxicity results or other reported trial outcomes, increasing target sample size).

Following each Albert Einstein Cancer Center DSMB meeting, provide the study leadership with written information concerning findings for the trial as a whole related to cumulative toxicities observed and any relevant recommendations related to continuing, changing, or terminating the trial. The study leadership will provide information on cumulative toxicities and relevant recommendations to the local principal investigators to be shared with their IRB's.

11. STATISTICAL CONSIDERATIONS

The primary aim of the study is to examine the difference in change of the outcome variables (Mena isoforms, cancer stem cells, and TMEM score) before treatment and 2-3 weeks after chemotherapy treatment begins. Two-sampled t-test will be used for this comparison. Appropriate transformation may be used to improved normality of the outcome variable. With 46 patients in stratum I and II (92 total), our study will have 80% power to detect a difference in change of 0.60SD of each outcome variable in each stratum, a moderate to large effect size[42]..As an exploratory analysis, we will also conduct multivariable linear regression model to examine the treatment difference in change in outcome variables while adjusting for other clinical and patient characteristics. We will also perform an exploratory analysis in 20 patients in stratum III.

12. REFERENCES

1. Mauri D, Pavlidis N, Ioannidis JP: Neoadjuvant versus adjuvant systemic treatment in breast cancer: a meta-analysis. J Natl Cancer Inst 97:188-94, 2005
2. Kaufmann M, von Minckwitz G, Mamounas EP, et al: Recommendations from an international consensus conference on the current status and future of neoadjuvant systemic therapy in primary breast cancer. Ann Surg Oncol 19:1508-16
3. Berruti A, Generali D, Kaufmann M, et al: International expert consensus on primary systemic therapy in the management of early breast cancer: highlights of the Fourth Symposium on Primary Systemic Therapy in the Management of Operable Breast Cancer, Cremona, Italy (2010). J Natl Cancer Inst Monogr 2011:147-51
4. Kaufmann M, Morrow M, von Minckwitz G, et al: Locoregional treatment of primary breast cancer: consensus recommendations from an International Expert Panel. Cancer 116:1184-91
5. Prowell TM, Pazdur R: Pathological Complete Response and Accelerated Drug Approval in Early Breast Cancer. N Engl J Med
6. Wolmark N, Wang J, Mamounas E, et al: Preoperative chemotherapy in patients with operable breast cancer: nine-year results from National Surgical Adjuvant Breast and Bowel Project B-18. J Natl Cancer Inst Monogr:96-102, 2001
7. Bear HD, Anderson S, Brown A, et al: The effect on tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide: preliminary results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. J Clin Oncol 21:4165-74, 2003
8. Green MC, Buzdar AU, Smith T, et al: Weekly paclitaxel improves pathologic complete remission in operable breast cancer when compared with paclitaxel once every 3 weeks. J Clin Oncol 23:5983-92, 2005
9. Buzdar AU, Valero V, Ibrahim NK, et al: Neoadjuvant therapy with paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide chemotherapy and concurrent trastuzumab in human epidermal growth factor receptor 2-positive operable breast cancer: an update of the initial randomized study population and data of additional patients treated with the same regimen. Clin Cancer Res 13:228-33, 2007
10. Hansen-Algenstaedt N, Stoll BR, Padera TP, et al: Tumor oxygenation in hormone-dependent tumors during vascular endothelial growth factor receptor-2 blockade, hormone ablation, and chemotherapy. Cancer Res 60:4556-60, 2000
11. Hotchkiss KA, Ashton AW, Mahmood R, et al: Inhibition of endothelial cell function in vitro and angiogenesis in vivo by docetaxel (Taxotere): association with impaired repositioning of the microtubule organizing center. Mol Cancer Ther 1:1191-200, 2002
12. Padera TP, Stoll BR, Tooredman JB, et al: Pathology: cancer cells compress intratumour vessels. Nature 427:695, 2004
13. Taghian AG, Abi-Raad R, Assaad SI, et al: Paclitaxel decreases the interstitial fluid pressure and improves oxygenation in breast cancers in patients treated with neoadjuvant chemotherapy: clinical implications. J Clin Oncol 23:1951-61, 2005
14. Ghebeh H, Lehe C, Barhoush E, et al: Doxorubicin downregulates cell surface B7-H1 expression and upregulates its nuclear expression in breast cancer cells: role of B7-H1 as an anti-apoptotic molecule. Breast Cancer Res 12:R48, 2010
15. Zhang P, Su DM, Liang M, et al: Chemopreventive agents induce programmed death-1-ligand 1 (PD-L1) surface expression in breast cancer cells and promote PD-L1-mediated T cell apoptosis. Mol Immunol 45:1470-6, 2008
16. Earl H.M VL, Hiller N et al: Neo-tAnGo: A neoadjuvant randomized phase III trial

of epirubicin/cyclophosphamide and paclitaxel ± gemcitabine in the treatment of women with high-risk early breast cancer (EBC): First report of the primary endpoint, pathological complete response (pCR). J Clin Oncol 27:(suppl; abstr 522), 2009

17. Alvarez RH BG, Hsu L, Cristofanilli M, Esteva FJ, Pusztai L, Buzdar AU, Hortobagyi GN, Valero V: Clinical Outcome of Two Sequences of Administering Paclitaxel (P) and Anthracyclines (A) as Primary Systemic Therapy (PST) and Adjuvant Chemotherapy (ACT) in Breast Cancer (BC) Patients: A Retrospective Analysis from the M. D. Anderson Cancer Center (MDACC).
. J Clin Oncol 28:(suppl; abstr 548), 2010

18. Roussos ET, Goswami S, Balsamo M, et al: Mena invasive (Mena(INV)) and Mena11a isoforms play distinct roles in breast cancer cell cohesion and association with TMEM. Clin Exp Metastasis 28:515-27

19. Goswami S, Philippar U, Sun D, et al: Identification of invasion specific splice variants of the cytoskeletal protein Mena present in mammary tumor cells during invasion in vivo. Clin Exp Metastasis 26:153-9, 2009

20. Kedrin D, Gligorijevic B, Wyckoff J, et al: Intravital imaging of metastatic behavior through a mammary imaging window. Nat Methods 5:1019-21, 2008

21. Kedrin D, Wyckoff J, Sahai E, et al: Imaging tumor cell movement in vivo. Curr Protoc Cell Biol Chapter 19:Unit 19 7 2007

22. Roussos ET, Keckesova Z, Haley JD, et al: AACR special conference on epithelial-mesenchymal transition and cancer progression and treatment. Cancer Res 70:7360-4, 2010

23. Goswami S, Sahai E, Wyckoff JB, et al: Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. Cancer Res 65:5278-83, 2005

24. Wang W, Goswami S, Lapidus K, et al: Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. Cancer Res 64:8585-94, 2004

25. Condeelis J, Singer RH, Segall JE: The great escape: when cancer cells hijack the genes for chemotaxis and motility. Annu Rev Cell Dev Biol 21:695-718, 2005

26. Gertler F, Condeelis J: Metastasis: tumor cells becoming MENAcing. Trends Cell Biol 21:81-90, 2011

27. Wang W, Wyckoff JB, Goswami S, et al: Coordinated regulation of pathways for enhanced cell motility and chemotaxis is conserved in rat and mouse mammary tumors. Cancer Res 67:3505-11, 2007

28. Roussos ET, Condeelis JS, Patsialou A: Chemotaxis in cancer. Nat Rev Cancer 11:573-87, 2011

29. Philippar U, Roussos ET, Oser M, et al: A Mena invasion isoform potentiates EGF-induced carcinoma cell invasion and metastasis. Dev Cell 15:813-28, 2008

30. Roussos ET, Wang Y, Wyckoff JB, et al: Mena deficiency delays tumor progression and decreases metastasis in polyoma middle-T transgenic mouse mammary tumors. Breast Cancer Res 12:R101, 2010

31. Roussos ET, Balsamo M, Alford SK, et al: Mena invasive (MenaINV) promotes multicellular streaming motility and transendothelial migration in a mouse model of breast cancer. J Cell Sci 124:2120-31, 2011

32. Bear JE, Svitkina TM, Krause M, et al: Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. Cell 109:509-21, 2002

33. Shapiro IM, Cheng AW, Flytzanis NC, et al: An EMT-driven alternative splicing program occurs in human breast cancer and modulates cellular phenotype. PLoS Genet 7:e1002218, 2011

34. Roussos ET, Goswami S, Balsamo M, et al: Mena invasive (Mena(INV)) and Mena11a isoforms play distinct roles in breast cancer cell cohesion and association with TMEM. Clin Exp Metastasis 28:515-27, 2011

35. Oktay MH, Gertler FB, Liu YF, et al: Correlated immunohistochemical and cytological assays for the prediction of hematogenous dissemination of breast cancer. J Histochem Cytochem 60:168-73

36. Robinson BD, Sica GL, Liu YF, et al: Tumor microenvironment of metastasis in human breast carcinoma: a potential prognostic marker linked to hematogenous dissemination. Clin Cancer Res 15:2433-41, 2009

37. Loi S, Symmans WF, Bartlett JM, et al: Proposals for uniform collection of biospecimens from neoadjuvant breast cancer clinical trials: timing and specimen types. Lancet Oncol 12:1162-8, 2011

38. Fumagalli D, Bedard PL, Nahleh Z, et al: A common language in neoadjuvant breast cancer clinical trials: proposals for standard definitions and endpoints. Lancet Oncol 13:e240-8, 2012

39. Hatzis C, Sun H, Yao H, et al: Effects of tissue handling on RNA integrity and microarray measurements from resected breast cancers. J Natl Cancer Inst 103:1871-83

40. Krause M, Dent EW, Bear JE, et al: Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. Annu Rev Cell Dev Biol 19:541-64, 2003

41. Symmans WF, Peintinger F, Hatzis C, et al: Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 25:4414-22, 2007

42. Cohen j: Statistical Power Analysis for the Behavioral Sciences. 2nd ed.Hillsdale, New Jersey: Erlbaum
, 1988