THERAPEUTIC TARGETING OF ER BETA IN TRIPLE NEGATIVE BREAST CANCER

Protocol Number

Mayo Clinic Protocol Number: MC1831
TBCRC Protocol Number: TBCRC051

Protocol Chair

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Co-Investigators

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Statistician

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Supporters

Johns Hopkins University on behalf of the Translational Breast Cancer Research Consortium (TBCRC)

Coordinating Center

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THERAPEUTIC TARGETING OF ERβ IN TRIPLE NEGATIVE BREAST CANCER

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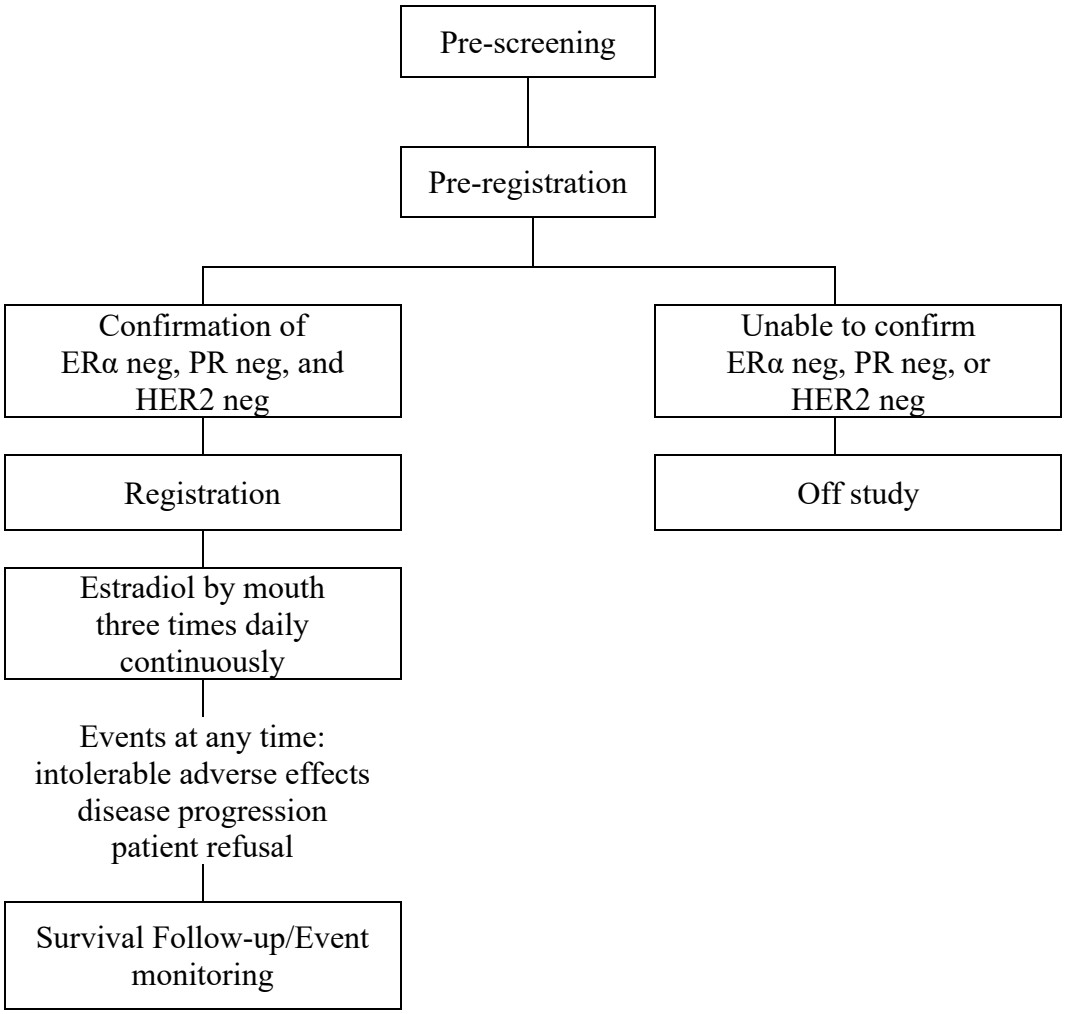
SCHEMA



Cycle length = 28 days ±3 days

Generic name: estradiol Brand name(s): Cenestin, Enjuvia, Estrace, Femtrace, Ogen, Premarin, etc. Mayo Abbreviation: estradiol Availability: Commercial

1. STUDY DESIGN/SUMMARY

This is a phase II study to determine the anti-tumor activity of estradiol in women with locally advanced or metastatic TNBC that expresses ERβ (>25% moderate or strong nuclear staining) and who have prior receipt of taxane and anthracycline based chemotherapy.

The study is divided into pre-screening, pre-registration phase and a registration phase:

Pre-Screening: Patients with a history of locally advanced or metastatic breast cancer that is ERα negative (<1% nuclear staining) and HER2 negative and who have received at most 3 prior chemotherapy regimens for treatment of metastatic breast cancer may register for the pre-screening portion of this trial. Note, pateints with a history of metastatic ERα+ breast cancer are not eligible. An archived FFPE tumor tissue from a locally advanced or metastatic site is to be submitted to the Mayo Clinic CAP/CLIA-certified Anatomic Pathology Laboratory for ERβ testing. If an archived FFPE tumor tissue specimen from a locally advanced or metastatic site is not available, then a specimen from primary tumor must be submitted. If the specimen is determined to have >25% moderate or strong nuclear staining for ERβ, the patient will be eligible for Pre-registration.

Pre-Registration: Patients determined to meet the requirements of the pre-screening phase and who have not undergone a biopsy of metastatic disease in the year prior to pre-registration will undergo a standard of care tumor biopsy for assessment of ERα, PR, and HER2 as well as three (3) additional research cores (see Section 10.2 and lab manual for details for processing). The first core will be FFPE and processed locally for ERα, PR, and HER2 as well as sectioning of three (3) unstained slides submitted simultaneously to Mayo Anatomic Pathology. Patients determined to be either ERα positive (>1% nuclear staining) or HER2 positive (see Section 4.1.2 for definition) are ineligible and should proceed with standard of care systemic therapy directed towards these validated targets. Those patients with confirmed ERα negative (<1% nuclear staining) and HER2 negative can proceed to registration. Patients known to have >25% moderate or strong nuclear staining for ERβ demonstrated in the pre-screening phase, and who have had a tissue-based biopsy of metastatic disease in the year prior to pre-registration can move directly to registration if all of the following are true:

- tissue from this metastatic biopsy was determined to be ERα negative (<1% nuclear staining) and HER2 negative

sufficient archived tissue is available for translational studies (a minimum of 3 and ideally 10 unstained slides cut at 5 microns)

2. OBJECTIVES

2.1 Primary Objective

To assess the anti-tumor activity of estradiol in patients with locally advanced or metastatic TNBC that expresses ERβ (>25% moderate or strong nuclear staining).

2.2 Secondary Objectives

- To examine the safety profile of estradiol when administered at a dose of 2 mg tid to women with locally advanced or metastatic TNBC that expresses ERβ
- To examine the changes in phospho-ERβ, cystatins 1, 2, 4 and 5, phospho-Smad2/3 and Ki-67 in tumor biopsies taken before and after the first cycle of treatment

2.3 Exploratory Objectives

- To examine changes in plasma estradiol, serum cytokine and cystatin levels before/after 1 cycle of estradiol
- Analyze the global gene expression profiles of paired biopsies prior to and following 1 cycle of therapy
- To develop PDX that are ERα negative, HER2 negative and ERβ positive (Mayo only)
- To examine changes in the relative abundance of circulating immune cell populations after the first cycle of treatment and whether these changes differ with respect to whether the patient is still on treatment after 6 cycles of treatment or not.

3. BACKGROUND

Our preliminary data indicate that ERβ expression (defined as >25% cells staining positive for moderate or high intensity within the nucleus) is present in up to 30% of TN breast tumors and expression is maintained in the majority of matched metastatic tumor lesions. Activation of ERβ by estrogen results in potent anti-cancer activity in ERβ expressing TNBC models in part through a novel mechanism involving increased cystatin expression which inhibits canonical TGFβ signaling, as well as direct suppression of the NFκB pathway by ERβ itself. Furthermore, through the establishment of TNBC PDX models, we have shown that ERβ positivity is associated with substantially reduced tumor growth rates in estrogen supplemented mice compared to ERβ negative TN PDXs. Ellis et al demonstrated the safety and efficacy of low dose estradiol (2 mg tid) in the setting of advanced ERα+ breast cancer.(Ellis et al. 2009) Therefore, using this dose and schedule, we will perform a prospective phase II study of estradiol in women with TNBC that is centrally confirmed (Mayo Clinic CLIA certified laboratory) to express ERβ and who have prior progression on taxane and anthracycline based chemotherapy. While our preliminary data demonstrate that up to 30% of TNBCs stain positive for moderate or high nuclear staining of ERβ, the cut-off for ERβ is unknown as a predictor of response. Therefore, we will chose to enroll patients with >25% moderate or strong nuclear staining.

ERβ expression in triple negative human breast tumors: We have developed an ERβ antibody that has been validated for clinical use in the Mayo Clinic Immunostains clinical laboratory, and have defined the IHC scoring system for ERβ based on extent and intensity of staining. Specifically, the extent of staining is scored as: 0: less than 1% positive cells, 1: 1%-25%, 2: 26%-50%, 3: 51%-75% and 4: 76%-100% while the intensity of staining is scored as none (0), weak (1), moderate (2) or strong (3). The resulting scores are summed and grouped into 3 categories, namely, negative/low (0-2), moderate (3-5) and high (6-7).

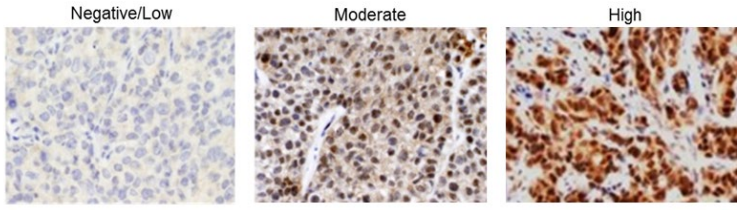


Figure 1. Representative images of an ERβ negative/low, moderate and high triple negative breast tumor.

Through the analysis of two separate cohorts of triple negative breast tumors totaling nearly 500 cases, we have demonstrated that ERβ protein is detectable at high levels in 5% of tumors and moderate levels in another 25% of tumors. Representative images of a negative/low, moderate and high expressing tumor are shown in Figure 1. Preliminary analysis of androgen receptor (AR) expression in these specimens indicates that ERβ+ and AR+ tumors do not significantly overlap suggesting that patients with ERβ positive TNBC represent a unique cohort. Importantly, we have also analyzed recurrent lesions from 27 patients whose primary tumors were ERβ positive and have demonstrated that ERβ protein expression is maintained at similar levels in the majority of these cases.

Biological effects of ERβ in triple negative breast cancer cells: The Hawse lab has developed ERβ expressing TNBC cell lines and have shown that treatment of these cells with estrogen or various ERβ specific agonists results in substantial inhibition of cell proliferation *in vitro*.(Reese et al. 2017; Reese et al. 2014) These inhibitory effects have been confirmed using multiple doses of a potent ERβ selective agonist, LY500307 (Figure 2). Inhibition of cell proliferation is a result of cell cycle arrest of TNBC cells following ligand mediated activation of ERβ (Figure 3). Molecular profiling of these cells has revealed that estrogen and ERβ selective agonists result in substantial up-regulation of a family of cysteine protease inhibitors known as the cystatins, specifically cystatin 1, 2, 4 and 5, at both the mRNA and protein level (data not shown).(Reese et al. 2018) Since cystatins are secreted proteins which elicit

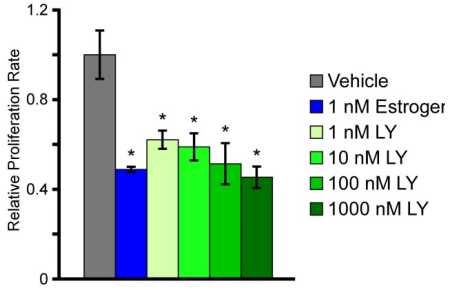


Figure 2. Effects of increasing concentrations of LY500307 on the proliferation rates of MDA-MB-231-ERβ cells relative to vehicle control treated cells.

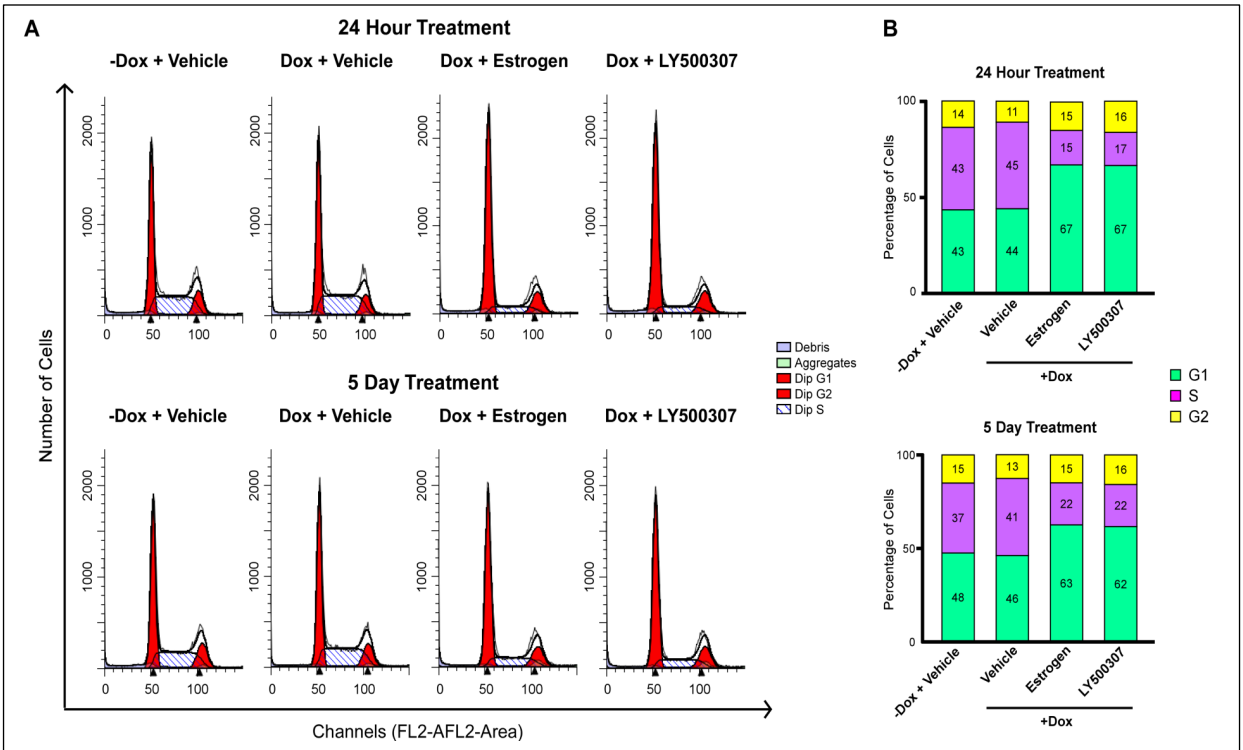


Figure 3. Cell Cycle Analysis of MDA-MB-231-ERβ with E2 or LY500307 treatment. Cells were treated for 24 hours or 5 days and flow cytometry was performed to determine cell cycle progression. (A) Raw peaks determined using ModFit software and (B) quantitation demonstrate an accumulation of cells in G1 and G2 following E2 or LY500307 treatment.

tumor suppressive effects in other types of cancers (Alvarez-Diaz et al. 2009; Shridhar et al. 2004; Briggs et al. 2010; Wegiel et al. 2009; Zhang et al. 2004; Jin et al. 2012)[3-8], we determined if conditioned media isolated from estrogen and ERβ agonist treated MDA-MB-231-ERβ expressing cells could inhibit the proliferation of parental MDA-MB-231 cells that do not express any form of the ERs. Furthermore, we determined if depletion of cystatins from conditioned media reversed this effect. Indeed, IgG depleted conditioned media isolated from 1nM estrogen and 100nM ERβ agonist treated MDA-MB-231-ERβ cells significantly inhibited the proliferation rates of parental MDA-MB-231 cells (Figure 4).

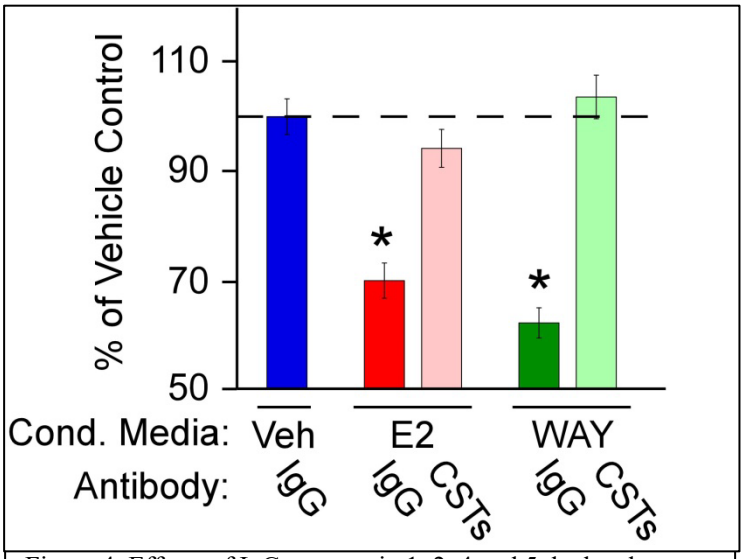


Figure 4. Effects of IgG or cystatin 1, 2, 4 and 5 depleted conditioned media isolated from 1nM E2 or 100nM WAY treated MDA-MB-231-ERβ cells on the proliferation rates of ERβ negative MDA-231 cells. Data are depicted relative to conditioned media isolated from vehicle treated cells following depletion with IgG.

These effects were completely reversed following depletion of cystatins from the media (Figure 4). We have also demonstrated that conditioned media isolated from estrogen and ERβ agonist treated cells inhibits TGFβ signaling as measured by the activity of a Smad

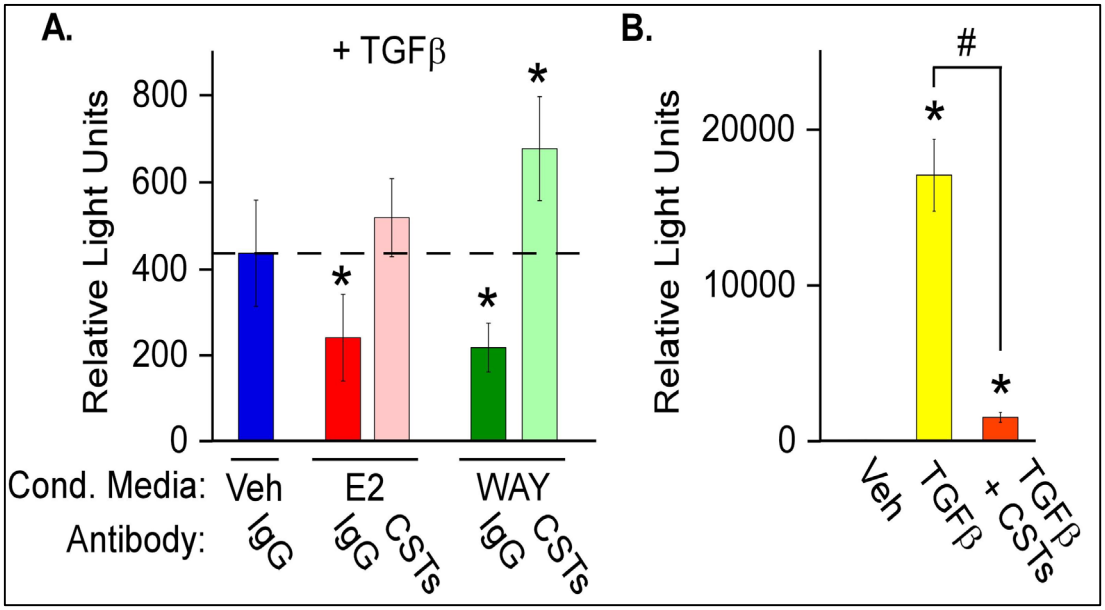


Figure 5. A). Conditioned media isolated from 1nM E2 and 100nM WAY treated MDA-MB-231-ERβ expressing cells significantly inhibits TGFβ mediated activation of a Smad Binding Element luciferase reporter construct. This inhibitory effect on the TGFβ pathway is lost when cystatins 1, 2, 4 and 5 (CSTs) are depleted from the media. B). A combination of recombinant CSTs 1, 2, 4 and 5 (0.125 mg/mL each) significantly inhibits TGFβ (1ng/mL) mediated activation of the Smad Binding Element reporter construct.

Binding Element (SBE) luciferase reporter construct (Figure 5A). Interestingly, depletion of cystatins from this conditioned media also reversed this effect (Figure 5A) suggesting that modulation of TGFβ signaling by ERβ may in part explain its tumor-suppressive effects.(Reese et al. 2018) Furthermore, pre-treatment of MDA-MB-231 cells with 0.125 µg/mL of recombinant cystatins 1, 2, 4 and 5 following transfection of the SBE significantly inhibited the ability of TGFβ to induce this reporter construct (Figure 5B). These observations are of particular significance since TGFβ signaling is known to promote tumor progression, metastasis and chemotherapy resistance in TNBC (Bhola et al. 2013; Bierie and Moses 2009; Giampieri et al. 2009; Matise, Pickup, and Moses 2009).

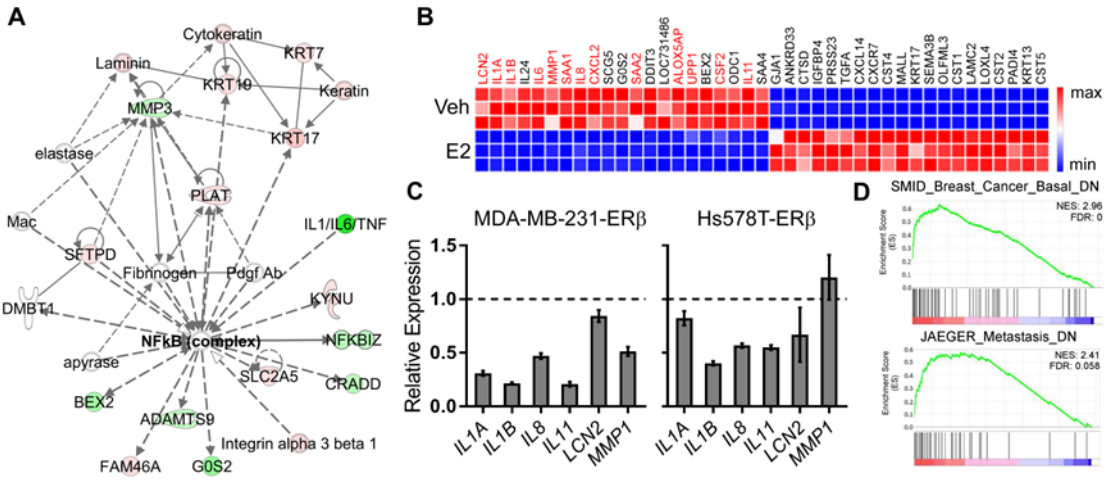


Figure 6. ERβ suppresses NFκB target genes in TNBC cells. (A) IPA analysis of the E2 regulated ERβ transcriptome in MDA-MB-231-ERβ cells indicated alterations in canonical NFκB signaling. **(B)** Heatmap depicting the top 20 E2 repressed (blue) and induced (red) genes. Gene names in red are known to be up-regulated by NFκB. **(C)** RT-PCR confirmation of E2-mediated suppression of NFκB target genes in 2 different ERβ expressing TNBC cell lines. **(D)** GSEA of the ERβ regulated transcriptome in MDA-MB-231-ERβ cells show positive correlation with signatures that are anti-metastatic and anti-basal-like in breast cancer.

Identification of the canonical NFκB pathway as a target of ERβ: Our gene expression studies and Ingenuity Pathway Analyses have also identified the canonical NFκB signaling pathway as being highly suppressed in response to E2 (Figure 6A). Indeed, the majority of down-regulated genes following E2 treatment consisted of well-known NFκB targets including multiple chemokines and cytokines (Figure 6B). Decreased expression of NFκB target genes following E2 treatment was confirmed by RT-PCR in both MDA-MB-231-ERβ and Hs578T-ERβ cell lines (Figure 6C). Gene set enrichment analysis (GSEA) showed that ERβ-regulated genes were positively correlated with signatures that are decreased in basal-like breast tumors or decreased in metastatic lesions suggesting that ERβ induces a more differentiated and less aggressive phenotype (Figure 6D). In parallel to these studies, we also performed chromatin immunoprecipitation assays for ERβ followed by sequencing (ChIPseq). We identified 26,896 ERβ binding sites across the genome in E2 treated MDA-MB-231-ERβ cells (Figure 7A and B), of which the large majority of binding sites resided at enhancer elements located within intergenic and intronic regions of chromatin (Figure 7C). Not surprisingly, the most

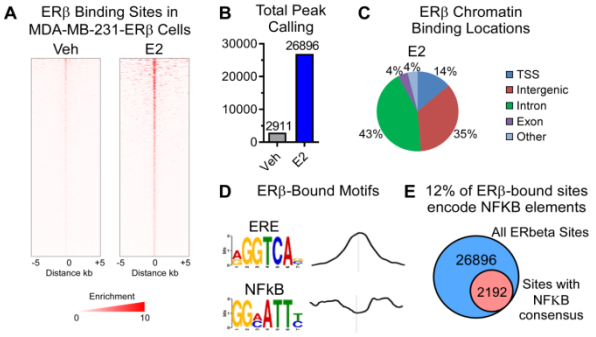


Figure 7. E2-induced ERβ cistrome in MDA-MB-231-ERβ cells. (A) Heatmap indicating E2-induced ERβ chromatin binding sites. **(B)** Number of ERβ binding sites identified and **(C)** their location in relation to specific gene regions. **(D)** Top motifs that are significantly enriched within ERβ binding locations. **(E)** Relative proportion of all ERβ bound chromatin sites that encode NFκB response elements.

common motif that ERβ associated with was a classic estrogen response element, but interestingly, ERβ was also enriched at NFκB binding motifs (Figure 7D). In fact, over 12% of all ERβ binding sites contained a consensus NFκB response element (Figure 7E), further implicating this pathway as a target of ERβ signaling.

Tumor suppressive effects of ERβ in vivo: We have also demonstrated that treatment of ERβ expressing MDA-MB-231 xenograft tumors with estrogen inhibits tumor growth relative to vehicle treated control animals (Figure 8).(Reese et al. 2018)

Figure 8. Estrogen suppresses MDA-MB-231-ERβ-Luc tumor growth *in vivo*. (A) Tumor progression was monitored at 8 weeks via IVIS2000 xenogen imaging and (B) average total flux was quantified. (C) Time to tumor doubling was determined between E2 treated animals and the placebo controls. The level of ERβ expression was determined in residual tumors at the time of sacrifice by (D) RT-PCR normalized to No Dox/Veh control cells, (E) western blotting and (F) immunohistochemistry.

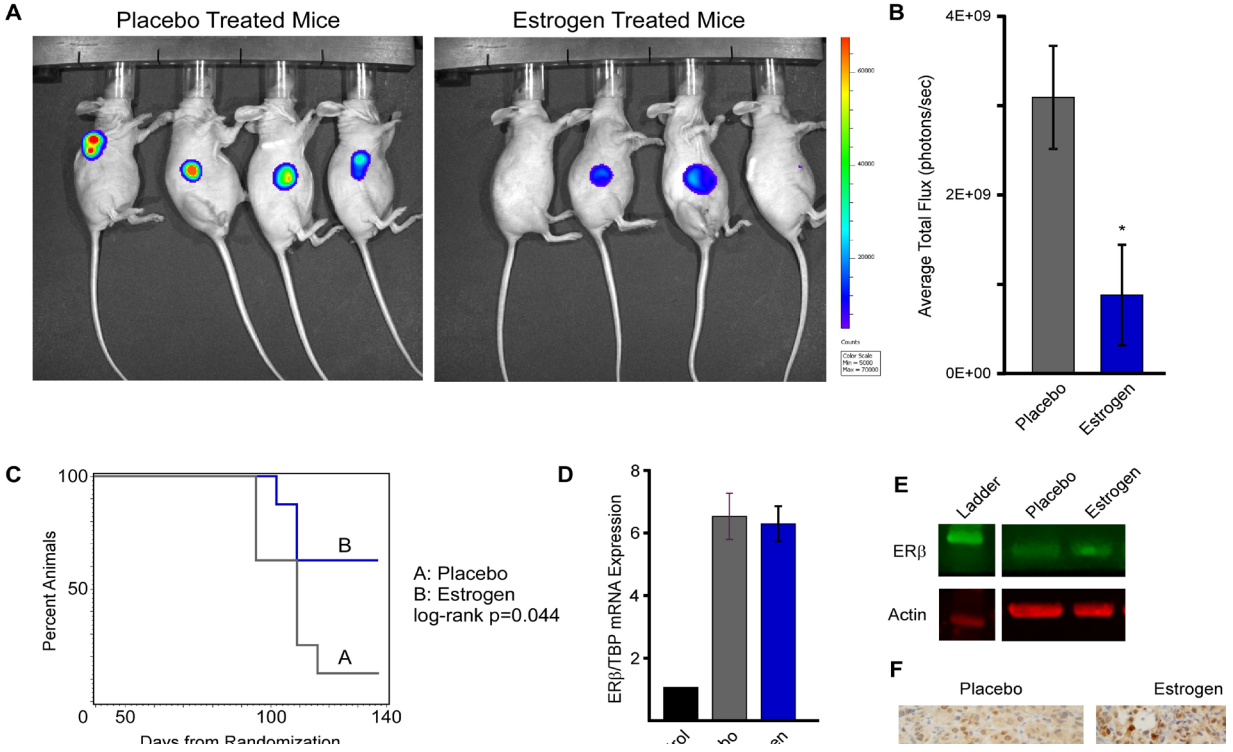
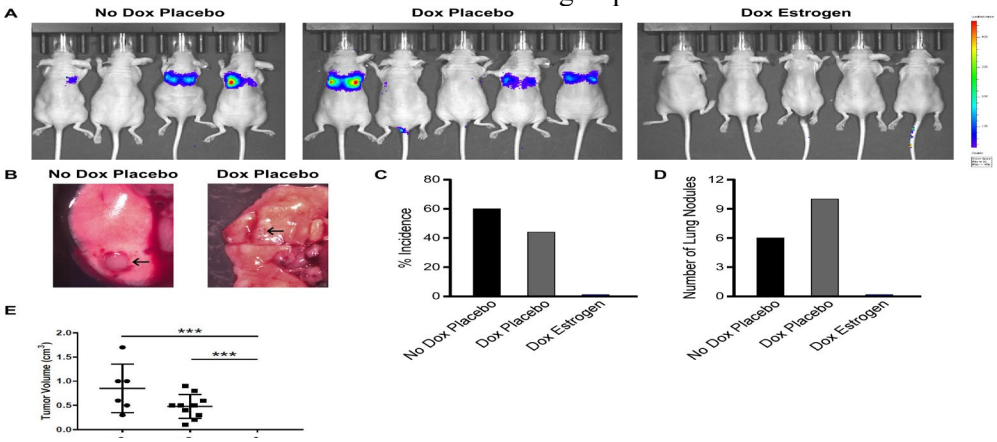
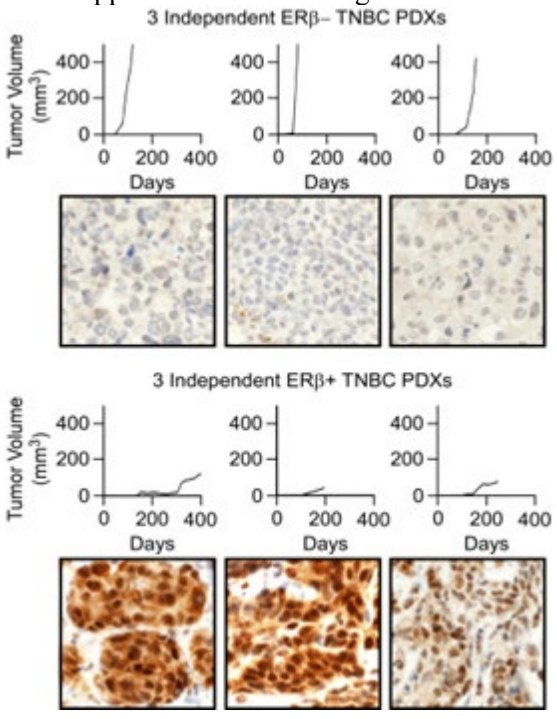


Figure 9. Estrogen treatment of ERβ-positive TNBC prevents lung tumor metastasis *in vivo*. OVX mice challenged with MDA-MB-231-ERβ-Luciferase cells via tail vein injection and randomized to treatment. (A) Presence of lung metastasis at week 8 via IVIS2000 xenogen imaging. At the termination of the study, lung dissections revealed the (B) presence of nodules in the no dox placebo and dox placebo groups (10x magnification). (C) Percent incidence, (D) number of lung nodules and (E) average tumor volume were also measured for each treatment group.



Similar effects have been observed in a second TNBC cell model system (Hs578T) (data not shown). Estrogen treatment of mice was also shown to completely block the development of metastatic lesions in nude mice following tail vein injection of ERβ expressing MDA-MB-231 cells (Figure 9). Using PDX models established from women with newly diagnosed high-risk breast cancer who were enrolled in a prospective neoadjuvant study at Mayo Clinic, we have now identified both ERβ+ and ERβ- triple negative breast tumors. Interestingly, during establishment of these PDXs in estrogen treated NOD Scid mice, ERβ+ tumors exhibit reduced growth rates compared to ERβ- tumors. Representative growth curves for three ERβ- and three ERβ+ PDX are shown in Figure 10. These data further support the hypothesis that ERβ is a potent tumor suppressor and that therapeutic targeting of this receptor may elicit anti-cancer effects in patients with ERβ+ TNBC.

Figure 10. Representative growth curves of ERβ- and ERβ+ TNBC PDXs in NOD SCID mice supplemented with estrogen.



4. PARTICIPANT SELECTION

4.1 Pre-Screening Inclusion Criteria (Step 0)

4.1.1 Women of age ≥18 years.

4.1.2 History of locally advanced or metastatic breast cancer that is ERα negative (<1% nuclear staining) and HER2 negative (see note below)

NOTE: HER2 Negative Disease per 2018 ASCO/CAP guidelines, **one** of the following must apply:

1) 0 or 1+ by IHC and not amplified by ISH

2) 0 or 1+ by IHC and ISH not done

3) 2+ by IHC and ISH results are: <6.0 HER2 signals/cell with HER2/CEP17 ratio <2.0

4) IHC not done and not amplified by ISH

4.1.3 ≤3 prior chemotherapy regimens for treatment of metastatic breast cancer

NOTE: Prior use of monoclonal antibodies targeting PD1, PDL1 is allowed (if administered as monotherapy it is not counted as a chemotherapy regimen).

4.1.4 ECOG Performance status 0 or 1 (see Appendix A)

4.1.5 Willing to submit a biopsy specimen from locally recurrent or metastatic site (or primary if metastatic site not available) of breast cancer for ERβ staining to Mayo Clinic Anatomic Pathology.

4.1.6 No prior history of metastatic ERα positive breast cancer (≥1%)

4.2 Pre-Registration Inclusion Criteria (Step 1)

4.2.1 Presence of moderate or strong nuclear ERβ staining in >25% of cells in specimen submitted during Pre-Screening Step.

4.2.2 For patients who did not have a biopsy or lacking ERα, PR, and HER2 results from a locally advanced or metastatic site performed ≤12 months prior to Pre-Registration: Willing to undergo a standard of care biopsy of locally recurrent or metastatic breast cancer for ERα, PR, and HER2 as well as additional research cores.

4.2.3 Measurable or non-measurable disease as defined by RECIST criteria (Section 11.2) that will be assessed using imaging-based evaluations (Section 11.3).

NOTE: The tumor lesion biopsied during the pre-registration period is not considered measurable disease nor a target lesion.

4.2.4 If history of brain metastases must meet the following criteria:

- Patients with a history of brain metastases are eligible only if they are asymptomatic and have stable disease for ≥3 months, including <28 days prior to Pre-Registration.
- Not receiving steroids for brain metastases

4.2.5 ECOG Performance status 0 or 1 (see Appendix A)

4.2.6 ≤3 prior chemotherapy regimens for treatment of metastatic breast cancer.

NOTE: Prior **use** of monoclonal antibodies targeting PD1, PDL1 is allowed

4.2.7 Women must be postmenopausal.

NOTE: Postmenopausal status is verified by:

1) Prior bilateral surgical oophorectomy, or
2) Age ≥60 years, or
3) Age <60 years with no menses for >1 year with estradiol levels within postmenopausal range, according to institutional standard

4.2.8 Able to swallow oral medications.

4.2.9 Willingness to stop use of strong inducers or inhibitors of CYP3A4 prior to registration.

NOTE: Use of strong inducers or inhibitors is allowed during **Pre-Registration** as long as patient will complete course prior to Registration.

4.3 Pre-Registration Exclusion Criteria

4.3.1 Uncontrolled intercurrent illness including, but not limited to:

- Ongoing or active infection
- Symptomatic congestive heart failure
- Unstable angina pectoris
- Uncontrolled symptomatic cardiac arrhythmia
- Uncontrolled hypertension (defined as blood pressure >160/90)

4.3.2 Any of the following co-morbid conditions:

- DVT/PE ≤12 months prior to pre-registration
  Note: Patients who are on anticoagulant therapy for maintenance are eligible as long as the DVT and /or PE occurred >6 months prior to Pre-Registration, and there is no evidence for active thrombosis (either DVT or PE).
- stroke ≤6 months prior to pre-registration
- two or more episodes of DVT and/or PE ≤5 years prior to pre-registration
- abnormal uterine bleeding ≤6 months prior to pre-registration
- history of coagulopathy

4.3.3 Other active second malignancy other than non-melanoma skin cancers within 3 years prior to pre-registration.
NOTE: A second malignancy is not considered active if all treatment for that malignancy is completed and the patient has been disease-free for ≥3 years prior to pre-registration.

4.4 Registration Inclusion Criteria (Step 2)

4.4.1 For patents who had a biopsy taken from a metastatic site ≤12 months prior to Pre-Registration: Confirmation from the local lab that the tumor from this biopsy was ERα negative (<1% nuclear staining) and HER2 negative.

4.4.2 For patients who underwent a pre-registration biopsy: Histologic confirmation from local lab that tumor is ERα negative (<1% nuclear staining), and HER2 negative (see prior note in Section 4.1.2).

4.4.3 Laboratory values ≤14 days prior to registration:

- Hemoglobin ≥8 g/dL
- Platelet Count ≥75,000/mm^3
- Creatinine ≤1.5 x upper limit of normal ULN
- Total Bilirubin ≤1.5 x upper limit of normal (ULN)
- AST/SGOT ≤2.5 x upper limit of normal (ULN)*

*For patients with liver metastasis: ≤5 x upper limit of normal (ULN)

4.5 Registration Exclusion Criteria

4.5.1 None of the following therapies are allowed ≤14 days prior to registration*:

- Chemotherapy
- Immunotherapy
- Biologic therapy
- Hormonal therapy
- Monoclonal antibodies
- Anti-HER2 or other "targeted" (e.g. mTOR) therapy

***NOTE**: Any adverse events derived from these therapies must be ≤Grade 2 prior to starting study therapy (exception: alopecia).

4.6 Inclusion of Underrepresented Populations

Individuals of all races and ethnic groups are eligible for this trial. There is no bias towards age or race in the clinical trial outlined. This trial is open to the accrual of women.

5. REGISTRATION PROCEDURES

5.1 Pre-Screening and Pre-Registration (Step 0 and Step 1)

5.1.1 TBCRC Institutions other than Mayo Clinic

To pre-screen or pre-register a patient, fax ([redacted]) a completed eligibility checklist to the Mayo Clinic Cancer Center (MCCC) Registration Office between 8 a.m. and 4:30 p.m. Central Time Monday through Friday excluding holidays.

5.1.2 Mayo Clinic Institutions

To pre-screen or pre-register a patient, access the Mayo Clinic Cancer Center (MCCC) web page and enter the registration/randomization application. The registration/ randomization application is available 24 hours a day, 7 days a week. Back up and/or system support contact information is available on the website. If unable to access the website, call the MCCC Registration Office at [redacted] between the hours of 8 a.m. and 5:00 p.m. Central Time (Monday through Friday excluding holidays).

The instructions for the registration/randomization application are available on the MCCC web page ([redacted]) and detail the process for completing and confirming patient registration. Prior to initiation of protocol treatment, this process must be completed in its entirety and an MCCC subject ID number must be available as noted in the instructions. It is the responsibility of the individual and institution registering the patient to confirm the process has been successfully completed prior to release of the study agent. Patient registration via the registration/randomization application can be confirmed in any of the following ways:

- Contact the MCCC Registration Office [redacted] If the patient was fully registered, the MCCC Registration Office staff can access the information from the centralized database and confirm the registration.
- Refer to “Instructions for Remote Registration” in section “Finding/Displaying Information about A Registered Subject.”

5.1.3 All Institutions:

5.1.3.1 Verification

Prior to accepting the pre-screening/pre-registration, the registration application will verify the following:

- IRB approval at the registering institution
- Patient pre-registration eligibility
- Existence of a signed consent form
- Existence of a signed authorization for use and disclosure of protected health information.

5.1.3.2 Pre-screening tests/procedures

Pre-screening/pre-registration tests/procedures (see Section 6) must be completed within the guidelines specified on the test schedule.

5.1.3.3 Biospecimen Banking - Patient Permissions

At the time of registration, the following will be recorded:

- Patient has/has not given permission to store and use his/her information and sample(s) for future research to learn about, prevent, or treat cancer.
- Patient has/has not given permission to store and use his/her sample(s) for future research to learn, prevent, or treat other health problems.
- Patient has/has/not given permission to have coded genetic information and coded medical information placed in password protected, secured database for research analyses.
- Patient has/has not given permission for study doctor or someone from the research team at the study doctor's site to contact them about future participation in more research

5.2 Registration Procedures (Step 2)

5.2.1 TBCRC Institutions other than Mayo Clinic

5.2.1.1 Registration

To register a patient, fax ([redacted]) a completed eligibility checklist to the Mayo Clinic Cancer Center (MCCC) Registration Office between 8 a.m. and 4:30 p.m. Central Time Monday through Friday.

5.2.1.2 Correlative Research

A mandatory correlative research component requiring blood and tissue (see Section 10) is part of this study. The patient will be registered onto this component by the MCCC Registration Office.

5.2.2 Mayo Clinic Institutions

5.2.2.1 Registration

To register a patient, access the Mayo Clinic Cancer Center (MCCC) web page and enter the registration/randomization application. The registration/ randomization application is available 24 hours a day, 7 days a week. Back-up and/or system support contact information is available on the Web site. If unable to access the Web site, call the MCCC Registration Office at [redacted] between the hours of 8 a.m. and 5:00 p.m. Central Time (Monday through Friday).

The instructions for the registration/randomization application are available on the MCCC web page ([redacted]) and detail the process for completing and confirming patient registration. Prior to initiation of protocol treatment, this process must be completed in its entirety and a MCCC subject ID number must be available as noted in the instructions. It is the responsibility of the individual and institution registering the patient to confirm the process has been successfully completed prior to release of the study agent. Patient registration via the registration/randomization application can be confirmed in any of the following ways:

- Contact the MCCC Registration Office [redacted] If the patient was fully registered, the MCCC Registration Office staff can access the information from the centralized database and confirm the registration.
- Refer to "Instructions for Remote Registration" in section "Finding/Displaying Information about A Registered Subject."

5.2.2.2 Correlative Research

A mandatory tissue and blood correlative research component is part of this study. The patient will be automatically registered onto this component (see Section 10).

5.2.3 All Institutions

5.2.3.1 Documentation

Documentation of IRB approval must be on file in the MCCC Registration Office before an investigator may register any patients.

In addition to submitting initial IRB approval documents, ongoing IRB approval documentation must be on file (no less than annually) at the Registration Office (fax: [redacted]). If the necessary documentation is not submitted in advance of attempting patient registration, the registration will not be accepted and the patient may not be enrolled in the protocol until the situation is resolved.

When the study has been permanently closed to patient enrollment, submission of annual IRB approvals to the Registration Office is no longer necessary.

5.2.3.2 Verification

Prior to accepting the registration, registration/randomization application will verify the following:

- IRB approval at the registering institution
- Patient eligibility
- Existence of a signed consent form
- Existence of a signed authorization for use and disclosure of protected health information

5.2.3.3 Biospecimen Banking - Patient Permissions

At the time of registration, the following will be recorded:

- Patient has/has not given permission to store and use his/her information and sample(s) for future research to learn about, prevent, or treat cancer.
- Patient has/has not given permission to store and use his/her sample(s) for future research to learn, prevent, or treat other health problems.
- Patient has/has/not given permission to have coded genetic information and coded medical information placed in password protected, secured database for research analyses.
- Patient has/has not given permission for study doctor or someone from the research team at the study doctor's site to contact them about future participation in more research

5.2.3.4 Treatment on this protocol must commence at a TBCRC institution under the supervision of a medical oncologist.

5.2.3.5 Treatment cannot begin prior to registration and must begin ≤7 days after registration.

5.2.3.6 Pretreatment tests/procedures (see Section 6) must be completed within the guidelines specified on the test schedule. Prior to registration (during pre-registration period) a research biopsy of metastatic breast tissue must be taken for central pathologic review.

5.2.3.7 All required baseline symptoms must be documented and graded.

5.2.3.8 Blood draw kit is available on site for this patient.

5.2.3.9 Descriptive Factor (to be recorded on On Study Form)
Tissue submission type: New biopsy at time of pre-registration vs. existing tissue from a metastatic site ≤12 months old

NOTE: Participants MUST be registered with the Lead Institution prior to the start of protocol treatment. **For sites outside of Mayo Clinic, registration can only be conducted during the business hours of 8 a.m. and 4:30 p.m. Central Time, Monday through Friday (excluding holidays).**

Note: Same day treatment registrations will only be accepted with prior notice and discussion with the Lead Institution.

6. STUDY CALENDAR

Parameter	ERβ screening (Pre-Screening)[1]	Prior to Pre-registration	Pre-registration period (prior to registration)	Prior to Day 1 of each treatment cycle[2]	End of treatment (EOT) for any reason[3]
Window	**N/A**	**≤21 days**	**≤28 days**	**±3 days**	**N/A**
CLINICAL EVALUATIONS:					
History and Physical		X		X	X
Height		X			
Vital signs and Weight				X	X
Performance status		X		X	X
LAB/RADIOLOGIC EVALUATIONS:					
Hematology (CBC/diff incl ANC, PLT)			X	X	X
Comprehensive Metabolic Panel[4]			X	X	X
Imaging/tumor measurements/ evaluation of indicator lesions[5],[6]			X	X	X
CORRELATIVE STUDIES: (See Section 10)					
Tissue submission	X[1]		X	X[7]	X[8]
Tumor/Tissue Biopsy			X[9]	X[10]	
Blood samples				X[11]	X
ADDITIONAL INFORMATION:					
Adverse Events/Toxicity Assessment			X	X	X
Patient Study Drug Diary (Appendix B)				X	X

[1] Pre-screening for ERβ may occur at any time prior to Pre-Registration using archival tissue from a local recurrence or metastatic site (or primary if local recurrence/metastatic site not available) of breast cancer.

[2] Cycle length is 28 (±3) days. Labs and AEs completed prior to registration may be used for Cycle 1 Day 1 tests if obtained <14 days prior to registration. For subsequent cycles, labs, scans, tests and observations may be obtained ≤3 days prior to Day 1 of treatment.

[3] Visit should be completed as soon as possible after last treatment and prior to starting any other treatment. Treatment may end due to progressive disease, intolerable adverse events, patient refusal, etc. See Section 12

[4] Panel includes: albumin, AST, ALT, alk phos, t bili, BUN, fasting calcium, creatinine, fasting glucose, sodium

[5] Acceptable imaging modalities for measurable disease include: CT or MRI. PET scanning is allowed to complement CT scanning in assessment of progressive disease.

[6] Tumor measurements are required during the Pre-Registration period (Days -28 to -1), at completion of Cycles 2, 4, 6, and about every 8 weeks until disease progression. The same imaging method used at baseline must be used at all disease assessments and must include all target and non-target lesions recorded at baseline.

[7] Research biopsy prior to Cycle 2 (end of Cycle 1) only

[8] If clinical biopsy performed at progression, please submit tissue sample (10 unstained slides)

[9] Patients must have had a tissue-based biopsy of a local recurrence or metastatic site ≤12 months prior to Pre-Registration and residual tissue must be available for testing. If a tissue-based biopsy was not performed ≤12 months prior to Pre-Registration, a new biopsy must be performed. Tissue must be submitted for central review at Mayo Clinic per Section 10 and Lab Manual.

[10] Research biopsy required prior to Cycle 2 (end of Cycle 1) only

[11] Blood samples collected prior to treatment on C1D1; prior to Cycle 2 (end of Cycle 1); and EOT – See Section 10

7. TREATMENT PLAN

7.1 Biopsy and enrollment

7.1.1 Prescreening for ERβ

Patients with archived tumor tissue available from a prior biopsy of locally recurrent or metastatic breast cancer site (or primary if locally recurrent/metastatic site not available) must submit a minimum of three (3) unstained 5 micron slides and ideally ten (10) unstained slides to the study central laboratory (Mayo Clinic) for staining, review and assessment of ERβ prior to full pre-registration. (See Section 10 and Lab Manual for submission instructions.)

Patients with ERβ moderate or strong nuclear staining >25% will be considered eligible to proceed to Pre-Registration.

7.1.2 Pre-Registration

All patients will need to consent to the full study prior to the biopsy.

Patients for whom tissue from a biopsy of locally recurrent or metastatic disease is not available for assessment of ERα, PR, and HER2 obtained in the 12 months prior to Pre-Registration will undergo a standard of care tumor biopsy for assessment of ERα, PR, and HER2 as well as three (3) additional research cores (see Section 10 and Lab Manual for details for processing). The first core will be FFPE and processed locally for ERα, PR, and HER2 and sectioning of three (3) unstained slides (submitted simultaneously to Mayo Anatomic Pathology).

Patients determined to be either ERα positive (>1% nuclear staining) or HER2 positive (see Section 4.1.2 for definition) are ineligible and should proceed with standard of care systemic therapy directed towards these validated targets.

Patients with confirmed ERα alpha negative (<1% nuclear staining), HER2 negative disease may proceed to Registration.

7.1.3 Registration

Verify labs and remaining items for eligibility.

7.2 Agent Administration

Agent	Dose	Route	Day	Continuation Cycle
Estradiol	2 mg	1 tablet three times daily by mouth	Days 1-28 (continuous)	Every 28 days (±3 days)

Instruct patient to take medication as consistently as possible

Instruct patient not to consume grapefruit or grapefruit juice while on study drug.

If a dose is missed, skip that dose and take the next scheduled dose.

If the patient vomits after taking a dose, skip that dose and resume with next scheduled dose.

7.3 Concomitant Treatment and Supportive Care Guidelines

7.3.1 Full Supportive Care

Patients should receive full supportive care while on this study, including blood product support, antibiotic treatment, and treatment of other newly diagnosed or concurrent medical conditions. All blood products and concomitant medications such as antidiarrheals, analgesics, and/or antiemetics received from the first day of study treatment administration until 30 days after the final dose should be recorded in the patient's institutional medical records.

7.3.2 Vaginal hemorrhage (bleeding/spotting)

7.3.2.1 Management of Grade 1 vaginal hemorrhage

Patients should continue taking the current dose of estradiol, and clinicians should manage according to institutional standards of care.

Suggestions include:

Use of a short course of medroxyprogesterone -- Clinicians may consider the use of medroxyprogesterone, 5-10 mg per day for 5-10 days until vaginal bleeding stops. Consideration should be for use of the lowest dose and duration of medroxy-progesterone as the effects of medroxyprogesterone on the antitumor effects of estradiol in TNBC are uknown.

7.3.2.2 Management for Grade ≥2 vaginal hemorrhage

Temporarily discontinue estradiol and start short course (5-10 days) of medroxy-progesterone as described in Section 7.3.2.1 above.

If AE returns to ≤Grade 1, restart estradiol at next lower dose.

If AE does not return to ≤Grade 1 within 14 days, discontinue estradiol.

7.3.3 Participation in Other Clinical Trials

Patients participating in this clinical trial are not to be considered for enrollment in any other study involving pharmacologic agents whether for symptom control or therapeutic intent.

7.3.4 Radiation Therapy

Patients must terminate study treatment if they are to receive radiation therapy for palliative reasons to the indicator lesion (s), as it impacts upon assessing the primary endpoint.

8. EXPECTED ADVERSE EVENTS AND DOSING DELAYS/DOSE MODIFICATIONS

8.1 Treatment Administration

Estradiol is an oral formulation designed to be taken three times per day with water on a continuous basis.

8.2 Anticipated Adverse Events

Consult the package insert for the most current and complete information.

Less common but serious side effects include endometrial cancer, ovarian cancer, breast cancer, stroke, myocardial infarction, venous thrombosis and pulmonary embolism, dementia and gallbladder disease. Other side effects include hypercalcemia, visual abnormalities, elevated blood pressure, hypertriglyceridemia, hypothyroidism, edema, exacerbation of other conditions (i.e. endometriosis, asthma, diabetes mellitus, epilepsy, migraine or porphyria, systemic lupus erythematosus, hepatic hemangiomas), breast pain, irregular vaginal bleeding or spotting, stomach/abdominal cramps, bloating, nausea and vomiting.

8.3 Management of Adverse Events

Strictly follow the modifications in this table for the first **two** cycles, until individual treatment tolerance can be ascertained. Thereafter, these modifications should be regarded as guidelines to produce mild-to-moderate, but not debilitating, side effects. If multiple adverse events are seen, administer dose based on greatest reduction required for any single adverse event observed. Reductions or increases apply to treatment given in the preceding cycle and are based on adverse events observed since the prior dose.

8.4 Dose Modifications/Delays

8.4.1 Dose Levels

Dose Level	Estradiol
0*	2 mg three times per day
-1	1 mg three times per day
-2	0.5 mg three times per day
-3	Discontinue

*Level 0 is the starting dose

Dose Modificatins for Adverse Events Associated with Estradiol. Note, In the case of adverse events persisting ≥14 days but in patients experiencing clinical benefit, contact the overall PI.

A maximum of two dose reductions are allowed.

8.4.2 Table of Actions for Adverse Events Associated with Estradiol

CTCAE System/Organ/Class	Adverse Event	Grade	Action for Estradiol
Blood and lymphatic system disorders	Anemia	≥3	Omit until resolved or return to ≤Grade 2 Then resume at next lower dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 2, then discontinue
Gastrointestinal disorders	Nausea	≥3	Omit until resolved or return to ≤Grade 2 Then resume at current dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 1, then discontinue If AE recurs despite appropriate antiemetic therapy then resume at next lower dose
Gastrointestinal disorders	Vomiting	≥3	Omit until resolved or return to ≤Grade 2 then resume at current dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 1, then discontinue If AE recurs despite appropriate antiemetic therapy then resume at next lower dose
Investigations	Lymphocyte count decreased	≥3	Omit until resolved or return to ≤Grade 2 Then resume at next lower dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 2, then discontinue
	Neutrophil count decreased	≥4	Omit until resolved or return to ≤Grade 2 Then resume at next lower dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 2, then discontinue
	Platelet count decreased	≥3	Omit until resolved or return to ≤Grade 2 Then resume at next lower dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 2, then discontinue

CTCAE System/Organ/Class	Adverse Event	Grade	Action for Estradiol
Investigations	White blood cell decreased	≥3	Omit until resolved or return to ≤Grade 2 Then resume at next lower dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 2, then discontinue
Metabolism and nutrition disorders	Hypercalcemia	≥2	Omit until resolved or return to ≤Grade 1 or baseline Then resume at next lower dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 1, then discontinue
	Hyponatremia	≥2	Omit until resolved or return to ≤Grade 1 Then resume at next lower dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 1, then discontinue
Nervous system disorders	Ischemia, cerebrovascular	2	Omit and initiate antiplatelet or anticoagulant therapy per local guidelines If return to ≤Grade 1, then resume at next lower dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 1, then discontinue
	Stroke	≥2	Discontinue
	Transient ischemic attacks	≥2	Omit and initiate antiplatelet or anticoagulant therapy per local guidelines If return to ≤Grade 1, then resume at next lower dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 1, then discontinue
Reproductive system and breast disorders	Vaginal hemorrhage	≥2	Omit estradiol and initiate medroxyprogesterone per Section 7.3.2 If return to ≤Grade 1, then restart estradiol at next lower dose If estradiol is omitted for >14 days and the AE does not return to ≤Grade 1, then permanently discontinue estradiol

CTCAE System/Organ/Class	Adverse Event	Grade	Action for Estradiol
Vascular disorders	Thromboembolic event	≥2	Omit until appropriate anticoagulation therapy can be initiated and patient is medically stable If drug is omitted for ≥14 days and the AE does not return to ≤Grade 1, then discontinue Discontinue if event recurs (second episode) while on anticoagulation therapy
All other	Any event not specified above	≥3	Omit until resolved or return to ≤Grade 1 Then resume at next lower dose If drug is omitted for ≥14 days and the AE does not return to ≤Grade 1, then discontinue Discontinue if event recurs

8.5 Special Considerations

- The treating investigator may reduce a subject's dose for an AE of any grade/duration where s/he believes it to be in the best interests of the subject.
- Any consideration to modification of the above dose modification guidelines should be discussed with the Principal Investigator for approval or disapproval in advance.

8.6 Adverse Events to be Graded at End of Each Cycle of Treatment

Use NCI Common Terminology for Adverse Events (CTCAE) v.5.0

CTCAE System/Organ/Class (SOC)	**Adverse Event/Symptoms**	**Baseline**	**Each Evaluation**
Gastrointestinal disorders	# of stools at baseline	X	
	Diarrhea		X
	Nausea	X	X
	Vomiting	X	X
Investigations	Platelet count decreased	X	X
	Neutrophil count decreased	X	X
	White blood cell decreased	X	X
Metabolism and nutrition disorders	Hypercalcemia	X	X
	Hyponatremia	X	X
Reproductive system and breast disorders	Vaginal hemorrhage	X	X

9. DRUG FORMULATION/STORAGE/SUPPLY

9.1 Estradiol

Estradiol is an approved agent for the treatment of metastatic breast cancer. Estradiol is commercially available.

9.1.1 Background

Estradiol is the principal intracellular human estrogen and is substantially more potent than its metabolites, estrone and estriol at the receptor level. Estrogens act through binding to nuclear receptors in estrogen-responsive tissues. Circulating estrogens modulate the pituitary secretion of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), through a negative feedback mechanism.

9.1.2 Formulation

NOTE: Will depend on generic brand dispensed at local pharmacy.

Each tablet, for oral administration, must contain 0.5, 1 or 2 mg of estradiol.

9.1.3 Storage

Instruct patient to store at room temperature.

9.1.4 Administration

Refer to the treatment section (Section 7) for specific administration instructions.

9.1.5 Pharmacokinetic information

Distribution: Estrogens are widely distributed in the body and are generally found in higher concentrations in the sex hormone target organs. Estrogens circulate in the blood largely bound to sex hormone binding globulin (SHBG) and albumin.

Metabolism: Estrogens also undergo enterohepatic recirculation via sulfate and glucuronide conjugation in the liver, biliary secretion of conjugates into the intestine and hydrolysis in the gut followed by reabsorption.

Excretion: Estradiol, estrone, and estriol are excreted in the urine along with glucuronide and sulfate conjugates.

9.1.6 Potential Drug Interactions

In vitro and *in vivo* studies have shown that estrogens are metabolized partially by cytochrome P450 3A4 (CYP3A4). Therefore, inducers or inhibitors of CYP3A4 may affect estrogen drug metabolism. Inducers of CYP3A4 such as St. John's Wort preparations (Hypericum perforatum), phenobarbital, carbamazepine, and rifampin may reduce plasma concentrations of estrogens, possibly resulting in a decrease in therapeutic effects and/or changes in the uterine bleeding profile. Inhibitors of CYP3A4 such as erythromycin, clarithromycin, ketoconazole, itraconazole, ritonavir and grapefruit juice may increase plasma concentrations of estrogens and may result in side effects.

9.1.7 Known potential adverse events

Consult the package insert for the most current and complete information.

Less common but serious side effects include endometrial cancer, ovarian cancer, breast cancer, stroke, myocardial infarction, venous thrombosis and pulmonary embolism, dementia and gallbladder disease. Other side effects include hypercalcemia, visual abnormalities, elevated blood pressure, hypertriglyceridemia, hypothyroidism, edema, exacerbation of other conditions (i.e. endometriosis, asthma, diabetes mellitus, epilepsy, migraine or porphyria, systemic lupus erythematosus, hepatic hemangiomas), breast pain, irregular vaginal bleeding or spotting, stomach/abdominal cramps, bloating, nausea and vomiting.

9.2 Ordering

Patients will be given a prescription for estradiol to fill at the pharmacy of their choice.

9.3 Drug Accountability

Drug accountability will be performed via patient pill diaries and patient appointments.

10. CORRELATIVE/SPECIAL STUDIES

10.1 Summary Table of All Biospecimens

Specimen	When to submit				
	Pre-Screening	During Pre-Reg Period	Baseline (C1D1 pre-treatment)	End of Cycle 1 (prior to Cycle 2)	End of Treatment for any reason
Archived FFPE slides (Section 10.3.1 and Section 10.3.4)	Mandatory				Optional
Frozen and FFPE (and PDX)* tissue (Section 10.3.1 and Section 10.3.2)		Mandatory		Mandatory	
Blood for DNA (Section 10.2.1)			Mandatory		
Blood for other correlatives (Section 10.2.1)			Mandatory	Mandatory	Mandatory

*PDX tissue is only collected at Mayo Clinic in Rochester, Minnesota.

NOTE: Optional items are strongly recommended.

10.2 Blood Samples

10.2.1 Summary of the blood biospecimen collection schedule

NOTE: Blood collection is **mandatory** for this study at all timepoints specified.

	Cycle 1, Day 1 prior to start of treatment (Baseline)	At the end of Cycle 1 (prior to start of Cycle 2)	At end of treatment for any reason	Submit to:
Estradiol, cytokine, cystatin	X	X	X	Mayo Clinic (per lab manual)
CTC	X	X	X	Mayo Clinic
cfDNA	X	X	X	Mayo Clinic
SNP	X			Mayo Clinic
PBMC	X	X	X	Mayo Clinic
Thymidine Kinase	x	x	x	Mayo Clinic

Kits will be provided for blood collection.

Instructions for collection and shipping are in each kit.

See Lab Manual for instructions.

Samples should be collected and shipped Monday – Friday.
However, if the subject can only be seen on Fridays, please ensure that Saturday delivery is marked clearly on the shipping boxes and Mayo Clinic staff are notified.

10.2.2 Methods

10.2.2.1 Circulating Tumor Cell Capture and Phenotyping

The relative abundance of CTCs in the peripheral blood has strong correlation with tumor recurrence and distant metastases in both early stage and advanced breast tumors. We will prospectively isolate CTCs from blood specimens collected from study participants at three time points for phenotypic and possibly genotypic analyses.

One blood sample will be collected in the CellSave® Preservation Tube for CTC isolation using the Veridex CellSearch™ system (Janssen; Raritan, NJ). CellSave® contains an optimized preservative that stabilizes cells for up to 96 hours at room temperature. The CellSearch™ system ([redacted]) allows for the

immunomagnetic selection, fluorescence staining, concentration, and enrichment of CTCs for enumeration and analysis. The FDA cleared process uses the CellSearch™ Epithelial Cell Kit, which contains anti-EpCAM (epithelial cell adhesion molecule) Ferrofluid, a fluorescent nucleic acid dye (DAPI), and fluorescently labeled monoclonal antibodies specific for leukocytes (CD45) and epithelial cells (cytokeratins 8, 18, and 19) to identify CTCs as those that are cytokeratin positive, DAPI positive, and CD45 negative with the appropriate morphology. At least two-thirds of patients with metastatic breast cancer have >1 CTC per 7.5 mL blood assayed with this technology, and the enumeration of CTCs relative to a threshold of 5 CTCs per 7.5 mL blood is associated with clinical outcomes in this patient population[27-29]. The standard process has been modified to allow for CTC retrieval and subsequent assessment for additional protein markers of interest (e.g., Aurora A kinase, ER, p-SMAD5, and p-SOX2).

CTCs vary in the degree of EpCAM expression, so malignant cells in the circulation may be missed by the CellSearch technology. Toward that end, other methodologies that do not rely on EpCAM for capture are now available. Therefore, a second blood sample will be collected in the AccuCyte® Blood Collection Tube for CTC isolation using the AccuCyte® Platform (RareCyte; Seattle, WA). The blood collection tube contains an optimized preservative that stabilizes cells for up to 96 hours at ambient temperature. The AccuCyte® Platform ([redacted]) is a comprehensive, reproducible, and highly sensitive platform for enriching, identifying, and isolating CTCs for downstream molecular analyses (ref: Campton DE et al. BMC Cancer 2015. 15:360.). The system relies on density gradient centrifugation and allows for virtually complete harvesting of the buffy coat into a small volume for application to a microscopic slide without cell lysis or washing. Immunofluorescent staining for selected markers (e.g., cytokeratins, EpCAM, DAPI, CD45, HER2, ER, Aurora A kinase, p-SMAD5, and p-SOX2) is used for the efficient positive and negative identification of CTCs via automated scanning digital microscopy and image analysis. An integrated device provides for mechanically precise CTC retrieval for advanced genomic analyses, including next generation sequencing, on single or pooled CTCs.

10.2.2.2 Peripheral blood mononuclear cell (PBMC) phenotyping using CyTOF

Analyses of tumor-host immune interactions have largely focused on exploring interactions between cancer cells and immune cells at the tumor tissue level. Efforts to understand perturbations in the systemic immune response occurring in the peripheral blood, and their influence on prognosis and chemotherapy response have been limited. It has been observed that decreased levels of circulating effector immune cells (e.g., CD8+ CTLs) or increased levels of circulating immune suppressive cells (e.g., monocytes, regulatory T cells, myeloid-derived suppressor cells) are associated with disease progression, and that anticancer therapy has an impact on the relative abundance of specific immune cell populations detectable in peripheral blood. The availability of multiplexed technologies such as Cytometry by time-of-flight (CyTOF™) has greatly improved the ability to evaluate a large number of surface markers at the single cell level, allowing for deep characterization of circulating immune cell populations in peripheral blood specimens.

Cytometry by time-of-flight (CyTOF™, Fluidigm) or mass cytometry is a platform that uses mass spectrometry to allow the evaluation of over 35 simultaneous parameters at a

single-cell resolution. This technology uses nonradioactive non-biological metal isotopes as reporters tagged to monoclonal antibodies. Measurements based on mass spectrometry largely avoids the hurdles of interference and spectral overlap experienced with fluorochrome-based flow cytometry. This constitutes an ideal platform for peripheral blood immune monitoring given its ability to assess a large number of parameters and resolve small differences in a heterogeneous population of cells.

Mononuclear cells will be isolated from peripheral blood via gradient separation using a Ficoll sodium diatrizoate solution (Ficoll-PaqueTM PLUS, GE Healthcare). The resulting mononuclear single-cell suspension will be suspended in freezing media containing 10% dimethyl sulfoxide and stored in liquid nitrogen or -150°C freezer for analysis. Specimens will be stained in batches of 4-5 patients. Unstimulated live single-cell suspensions will be stained with a cocktail of metal-tagged antibodies designed to recognize 29 cell surface proteins optimized for immune monitoring of human peripheral blood. Nucleated cellular events will be identified using a DNA intercalator conjugated to natural abundance iridium (191Ir and 193Ir). Cisplatin (195Pt) will be used for dead-live cell discrimination and calibration beads containing natural abundance cerium (140/142Ce), europium (151/153Eu), holmium (165Ho), and lutetium (175/176Lu) will be used for normalization of acquired data. Our panel will be constructed using commercially available metal-conjugated antibodies (Fluidigm Corporation) and stained cells will acquired on the CyTOF2 (Fluidigm Corporation).

We will stain a minimum of 3 x 10^6 cells per specimen and acquire a minimum of 1 x 10^6 events on the CyTOF. We will initially perform high-level manual gating to exclude dead or apoptotic cells, debris, beads and doublets. Additional multiparametric analysis will be done using platforms such as Astrolabe Diagnostics, Spanning-tree Progression Analysis of Density-normalized Events (SPADE), and visualization of unbiased clustering of events using the t-SNE (t-Distributed Stochastic Neighbor Embedding) algorithm or viSNE.

10.2.2.3 Thymidine Kinase

Serum thymidine kinase activity measurements have shown changes in levels during progression and treatment of metastatic breast cancer and other cancers. Thymidine kinase 1 (TK1) is a key enzyme in DNA synthesis. TK1 catalyzes the conversion of thymidine to deoxythymidine monophosphate (dTMP), which is further phosphorylated to di- and triphosphates preceding its incorporation into DNA. Expression of TK1 is E2F-dependent and peaks in the S-phase of the cell cycle, and CDK4/6 inhibition prevents E2F-dependent transcription of TK1 (Thomas, 2016). Due to uncontrolled cellular proliferation and higher replication rates compared to normal tissues, solid tumors can secrete pathological levels of TK1 that can be detected in serum. Studies have shown that serum TK1 levels were predictive of response, progression free survival (PFS) and breast cancer-free survival in both the neoadjuvant and metastatic settings (all disease subtypes). This was recently demonstrated in a secondary analysis of the SWOG S0226 trial, where patients with baseline elevated TKI using the DiviTum® TK assay had significantly worse PFS [median 11.2 vs. 17.3 months, HR = 1.76; 95% confidence interval (CI; 1.43-2.16); P < 0.0001] and OS [median 30 vs. 58 months, HR = 2.38; 95% CI (1.91-2.98); P < 0.0001] compared to those with low TK1 levels. Furthermore, at serial timepoints, high versus low sTK1 had significantly worse

subsequent PFS and OS [at cycle 2: PFS HR = 1.70, 95% CI (1.34-2.17); P < 0.0001, OS HR = 2.51, 95% CI (1.93-3.26); P < 0.0001]. (Paoletti et al. Clin Canc Res 2021).

We will measure thymidine kinase activity (TKa) in serum samples obtained at baseline, end of cycle 1 and end of treatment using the DiviTum® TKa (DiviTum® TKa ELISA kit, Biovica Cat #950). TK activity is determined from a calibration curve established using TK1 standards. The units are expressed as DiviTum units (DuA). The DuA value is established so that 1 DuA is the TK activity of 1 pg/mL of standard recombinant TK under the standard DiviTum® TKa assay conditions.

10.3 Tissue Samples

10.3.1 Pre-screening: ERβ pretesting (mandatory)

Correlative Study (Section for more information)	Type of Tissue to Collect/ Send	Process at site? (Yes or No)	Temperature conditions for Storage /Shipping
Submission of archived tumor specimen from locally recurrent or metastatic breast cancer for ERβ staining	10 unstained slides (5 micron)	Cut sections at local site Send to Mayo Coordinator	Room Temperature (Unstained slides)

For patients who have registered for the prescreening phase and have archived FFPE tumor tissue from a locally advanced or metastatic site (or primary if locally recurrent/metastatic site not available) available for ERβ testing in the Mayo CAP/CLIA certified Anatomic Pathology Laboratory, the local site must submit 10 unstained sections (cut at 5 microns) per instructions in the Lab Manual.

Requirements for using archived tissue:

- Biopsy must have been tissue-based. Fine needle aspirations (FNA) are NOT allowed for this submission.
- Ideally 10 unstained sections or a minimum of 3 unstained sections (cut at 5 microns) must be available for submission.

If all the above conditions cannot be met, then the patient cannot proceed to pre-registration.

Results of testing (either positive or negative for ERβ), will be reported to the local study coordinator.

If the result is negative for ERβ, patient may be directed to another study or may choose to have a new biopsy to confirm the result.

If the result is positive for ERβ (>25% moderate or strong nuclear staining) patients should be encouraged to proceed to Pre-Registration phase.

10.3.2 Pre-Registration Tumor Tissue Collection

10.3.2.1 Tissue Collection for Patients undergoing Pre-Registration Biopsy ONLY

Correlative Study	Mandatory or Optional	Type of Tissue to Collect	Process at site? (Yes or No)	Temperature Conditions for Storage /Shipping	Assay to be performed
Core 1 (FFPE)	Mandatory	Fresh tumor biopsy for FFPE	Yes	Room Temperature (Unstained slides)	ER, PR, HER2 (tested locally) and 3 additional unstained sections (sent to Mayo Clinic)
Core 2 (FFPE)	Mandatory	Fresh tumor biopsy for FFPE	Yes-FFPE	Room Temperature	For example: Ki-67, phospho-ERβ, phospho-smad 2/3, Cystatins 1, 2, 4, and 5
Core 3 (Frozen)	Mandatory	Fresh tissue core (core) for flash freezing	Yes	Frozen/ dry ice	DNA and RNA sequencing
Core 4 (PDX for **Rochester patients only**)	Mandatory	Fresh tissue biopsy placed in media	Yes	Fresh	PDX

A total of 3 or 4 cores will be obtained as outlined in Table 10.3.2 (above). The first core will be FFPE and processed as a standard of care test for ERα, PR, and HER2 as well as sectioning of three (3) unstained slides submitted simultaneously to Mayo Anatomic Pathology.

Patients determined to be either ERα positive (>1% nuclear staining) or HER2 positive (see Section 4.1.2 for definition) are ineligible and should proceed with standard of care systemic therapy directed towards these validated targets.

Patients confirmed to have ERα negative (<1% nuclear staining), and HER2 negative disease. Mayo Anatomic Pathology will proceed with ERβ testing using the same tumor block; however, these results will not be used for eligilblity. Core number 2 (Research FFPE) will be stored in Mayo Clinic BAP and processed (along with biopsy samples from Cycle 1) at a later time for antibodies such as: phospho-ERβ, cystatins 1, 2, 4 and 5, phospho-Smad2/3 and Ki-67. Core number 3 will be frozen and processed later for DNA and RNA sequencing. Core number 4 (Mayo Rochester only) will be processed for PDX generation.

10.3.2.2 For Patients with Tumor Tissue Available from a Biopsy/Excision Performed ≤12 months Prior to Pre-Registration

Submit archived FFPE tissue from biopsy/excision performed ≤12 months prior to Pre-Registration.

- Biopsy must have been tissue-based. Fine needle aspirations (FNA) are NOT allowed for this submission.

- Ideally 10 unstained sections or a minimum of 3 unstained sections (cut at 5 microns) must be available for submission.

Note: If tissue-based biopsy of metastatic disease was performed ≤12 months prior to Pre-Registration, and there is sufficient archived tissue for a minimum of 3 and ideally 10 unstained slides cut at 5 microns, then a new biopsy is not required.

10.3.3 Guidelines for tissue acquisition and shipping

See Lab Manual for specific information

Image guided biopsies are recommended where feasible. The amount of tissue collected will follow the guidelines listed below. If a patient has more than one site of disease, only one site needs to be biopsied.

Skin/breast/chest wall: An incisional/excisional biopsy is preferred. If punch biopsy is performed, a minimum of 4 punch biopsies (≥5mm diameter).

Lymph node: A goal of 4-6 core biopsy specimens obtained using an 18-gauge needle or larger. Smaller or less samples will be obtained at the discretion of the physician performing the biopsy.

Liver: A goal of 4-6 core biopsy specimens obtained using an 18-gauge needle.

Lung: Because of the risk of pneumothorax associated with core needle biopsies of lung nodules, biopsies are not recommended.

Bone: Because the yield of malignant tissue from bone biopsies tends to be relatively low, if a patient has another accessible site of disease (i.e., skin, lymph node, liver, other metastatic lesions outside of the bone), that site should be biopsied preferentially. If bone is the only biopsy-accessible site, then a goal of 3-6 core biopsy specimens will be obtained using an 18-gauge needle.

A series of high throughput assays including exome sequencing, and RNA seq will be applied using tumor samples baseline (DNA and RNA seq) and after Cycle 1 (RNA).

10.3.3.1 Xenograft mice model (Patient derived xenograft [PDX]) – Pre-Reg only

In Mayo Clinic (Rochester) Subjects Only:

For Mayo Clinic patients in Rochester, MN, registered to TBCRC051/MC1831, tumor biopsy material from core number 4 will be immediately injected into NGS mice for the creation of human tissue xenografts (see IACUC A49111 and IACUC A00003279-17). The xenograft tumor samples will be used to determine the functional implications of tumor alterations identified from tumor sequencing and for future drug screening and cytotoxicity assay. These lines will be also useful for future research purposes, such as testing novel compounds and different regimens. All the tumor tissues from xenografts will be stored on Gonda 19-466E.

10.3.4 Research Biopsy for ALL Patients Obtained after Cycle 1:

NOTE: Tissue collection is mandatory for this study

Correlative Study	Mandatory or Optional	Type of Tissue to Collect	Process at site? (Yes or No)	Temperature Conditions for Storage/ Shipping	Planned Assays
Core 1 (FFPE)	Mandatory	Fresh tumor biopsy for FFPE	Yes to create FFPE	Room Temperature (Unstained slides)	Ki-67, phosphor ERβ, phospho-smad 2/3, Cystatins 1, 2, 4, and 5
Core 2 (FFPE)	Mandatory	Fresh tumor biopsy for FFPE	Yes to create FFPE	Room Temperature	DNA and RNA sequencing
Core 3 (Frozen)	Mandatory	Fresh tissue core for flash freezing	Yes	Frozen/ dry ice	Storage for sequencing later

See Lab Manual for specific instructions for collection/shipping

10.3.4.1 Evaluation of changes in phospho-ERβ, cystatins 1, 2, 4 and 5, phospho-Smad2/3 and Ki-67

As shown in the preliminary data, we have demonstrated that ligand mediated activation of ERβ leads to increased expression of cystatins 1, 2, 4 and 5 as well as suppression of phospho-Smad2/3 and downstream markers of canonical TGFβ signaling. Additionally, based on recent data that phosphorylation of tyrosine 36 of ERβ (but not total ERβ) in breast tumors is associated with activation of ERβ, we will use core biopsies obtained after Cycle 1 (along with the baseline biopsies) to assess for changes in the phosphorylation of ERβ Y36 and Smad2/3, as well as the expression levels of cystatins 1, 2, 4 and 5 and Ki67.

Our hypothesis is that estradiol will activate ERβ resulting in 1) down-regulation of Ki-67; 2) reduction in phospho-Smad2/3; 3) increase in phosphorylation (activation) of ERβ; and finally 4) increase in expression levels of cystatins 1, 2, 4, and 5.

Additionally, these core biopsies will be used for RNA sequencing, to assess and compare changes in gene expression comparing tumors sensitive and resistant to estradiol.

10.3.4.2 Frozen Tissue for Sequencing/Exome (Core 3)

Tumor DNA will be isolated from specimens obtained and baseline. DNA will be processed and plated at Mayo BAP lab. After the first 10 patients have been enrolled, DNA and RNA and RNA yields will be reviewed. If inadequate, the protocol will be changed to use one core for DNA and another core for RNA sequencing.

10.3.5 Tissue Submission at End of Treatment for Any Reason

This submission is optional if archived tissue is available.

Correlative Study (Section for more information)	Type of Tissue to Collect/ Send	Process at site? (Yes or No)	Temperature Conditions for Storage/ Shipping
Submission of archived tumor specimen from locally recurrent or metastatic breast cancer for ERβ staining	5 unstained slides (5 micron)	Cut sections at local site Send to Mayo Pathology Coordinator	Room Temperature (Unstained slides)

For patients who have enrolled in this study, and for whom a clinical biopsy is performed at time of suspected disease progression, and for whom archived FFPE tumor tissue from a metastatic site is available, the local site should submit 5 unstained sections (cut at 5 microns) per instructions in the Lab Manual

10.4 Other Samples and/or Devices

10.4.1 Patient Derived Xenograft (Mayo Clinic Rochester patients only)

Xenografts will be generated in [redacted] Her lab has already successfully generated a series of breast cancer models for all subtypes from both primary breast cancer as well as metastatic breast cancer using core biopsy or surgical samples. In this study, to generate individual tumor xenograft line for drug screening, drug cytotoxicity assay, specimen obtained during the biopsy will be immediately injected into NGS mice for the creation of human tissue xenografts. The xenograft tumor samples will be used to determine the functional implications of tumor alterations identified from tumor sequencing and for future drug screening and cytotoxicity assay. These lines will be also useful for future research purposes, such as testing novel compounds and different regimens. All the tumor tissues from xenografts will be stored on Gonda 19 466E.

10.5 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 Mayo Clinic 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.

10.6 Additional Information

Submission of data for Genome Wide Association Studies (GWAS) is not currently planned; however, subjects will be asked for permission in the informed consent process. A revision to the protocol and/or any regulatory approvals will be secured prior to any GWAS submission or inclusions in the future, if applicable.

10.7 Specimen Banking

The study Protocol Chair and collaborators have approval by the TBCRC to use all research bio-specimens collected during the conduct of this trial to address the research questions described in the protocol document. All future use of residual or repository specimens collected in this trial for purposes not prospectively defined will require review and approval by the TBCRC according to its established policies, whether the specimens are stored in a central site or at a local institution in a virtual repository.

Secondary use of biospecimens for new endpoints must be submitted to the TBCRC Central Office for possible review by the TBCRC Correlative Science Review Committee.

11. MEASUREMENT OF EFFECT

Response and progression will be evaluated in this study using the international criteria proposed by the revised Response Evaluation Criteria in Solid Tumors (RECIST) guidelines (version 1.1) Changes in the largest diameter of the tumor lesions and the short axis measurements in the case of lymph nodes are used in the RECIST guideline.

11.1 Schedule of Evaluations

For the purposes of this study, patients should be re-evaluated every 8 weeks (at the end of every 2 cycles) until progression.

11.2 Definitions of Measurable and Non-Measurable Disease

11.2.1 Measurable Disease

A non-nodal lesion is considered measurable if its longest diameter can be accurately measured as 2.0 cm with chest x-ray, or as ≥1.0 cm with CT scan, CT component of a PET/CT, or MRI.

A superficial non-nodal lesion is measurable if its longest diameter is ≥1.0 cm in diameter as assessed using calipers (e.g. skin nodules) or imaging. In the case of skin lesions, documentation by color photography, including a ruler to estimate the size of the lesion, is recommended.

A malignant lymph node is considered measurable if its short axis is ≥1.5 cm when assessed by CT scan (CT scan slice thickness recommended to be no greater than 5 mm).

Tumor lesions in a previously irradiated area are not considered measurable disease.

The tumor lesion biopsied during the pre-registration period is not considered measurable disease.

Lytic bone lesions, with an *identifiable soft tissue component*, evaluated by X-ray, CT (with bone windows) or MRI, *can be considered as measurable lesions* if the soft tissue component otherwise meets the definition of measurability described above

11.2.2 Non-Measurable Disease

All other lesions (or sites of disease) are considered non-measurable disease, including pathological nodes (those with a short axis ≥1.0 to <1.5 cm). Bone lesions, leptomeningeal disease, ascites, pleural/pericardial effusions, lymphangitis cutis/pulmonis, inflammatory breast disease, and abdominal masses (not followed by CT or MRI), are considered as non-measurable as well.

Note: 'Cystic lesions' thought to represent cystic metastases can be considered as measurable lesions, if they meet the definition of measurability described above. However, if non-cystic lesions are present in the same patient, these are preferred for selection as target lesions. In addition, lymph nodes that have a short axis <1.0 cm are considered non-pathological (i.e., normal) and should not be recorded or followed.

11.3 Guidelines for Evaluation of Measurable Disease

11.3.1 Measurement Methods:

- All measurements should be recorded in metric notation (i.e., decimal fractions of centimeters) using a ruler or calipers.
- The same method of assessment and the same technique must be used to characterize each identified and reported lesion at baseline and during follow-up. For patients having only lesions measuring at least 1 cm to less than 2 cm must use CT imaging for both pre- and post-treatment tumor assessments.

11.3.2 Acceptable Modalities for Measurable Disease:

- **Conventional CT and MRI:** This guideline has defined measurability of lesions on CT scan based on the assumption that CT slice thickness is 5 mm or less. If CT scans have slice thickness greater than 5 mm, the minimum size for a measurable lesion should be twice the slice thickness.

  As with CT, if an MRI is performed, the technical specifications of the scanning sequences used should be optimized for the evaluation of the type and site of disease. The lesions should be measured on the same pulse sequence. Ideally, the same type of scanner should be used and the image acquisition protocol should be followed as closely as possible to prior scans. Body scans should be performed with breath-hold scanning techniques, if possible.

- **PET-CT:** If the site can document that the CT performed as part of a PET-CT is of identical diagnostic quality to a diagnostic CT (with IV and oral contrast), then the CT portion of the PET-CT can be used for RECIST measurements and can be used interchangeably with conventional CT in accurately measuring cancer lesions over time.
- **Chest X-ray:** Lesions on chest x-ray are acceptable as measurable lesions when they are clearly defined and surrounded by aerated lung. However, CT scans are preferable.
- **FDG-PET:** FDG-PET scanning is allowed to complement CT scanning in assessment of progressive disease [PD] and particularly possible 'new' disease. A 'positive' FDG-PET scanned lesion is defined as one which is FDG avid with an update greater than twice that of the surrounding tissue on the attenuation corrected image; otherwise, an FDG-PET scanned lesion is considered 'negative.' New lesions on the basis of FDG-PET imaging can be identified according to the following algorithm:

  a. Negative FDG-PET at baseline with a positive FDG-PET at follow-up is a sign of PD based on a new lesion.

  b. No FDG-PET at baseline and a positive FDG-PET at follow-up:

  - If the positive FDG-PET at follow-up corresponds to a new site of disease confirmed by CT, this is PD.
  - If the positive FDG-PET at follow-up is not confirmed as a new site of disease on CT at the same evaluation, additional follow-up CT scans (i.e., additional follow-up scans at least 4 weeks later) are needed to determine if there is truly progression occurring at that site. In this situation, the date of PD will be the date of the initial abnormal PDG-PET scan.
  - If the positive FDG-PET at follow-up corresponds to a pre-existing site of disease on CT that is not progressing on the basis of the anatomic images, it is not classified as PD.

11.3.3 Measurement at Follow-up Evaluation:

- A subsequent scan must be obtained not less than 4 weeks following initial documentation of an objective status of either complete response (CR) or partial response (PR).
- In the case of stable disease (SD), follow-up measurements must have met the SD criteria at least once after study entry at a minimum interval of not less than 6-8 weeks (see Section 11.44).
- The cytological confirmation of the neoplastic origin of any effusion that appears or worsens during treatment when the measurable tumor has met criteria for response or stable disease is mandatory to differentiate between

response or stable disease (an effusion may be a side effect of the treatment) and progressive disease.

- Cytologic and histologic techniques can be used to differentiate between PR and CR in rare cases (e.g., residual lesions in tumor types such as germ cell tumors, where known residual benign tumors can remain.)

11.4 Measurement of Treatment/Intervention Effect

11.4.1 Target Lesions & Target Lymph Nodes

- Measurable lesions (as defined in Section 11.2.1) up to a maximum of 5 lesions representative of all involved organs, should be identified as "Target Lesions" and recorded and measured at baseline. These lesions can be non-nodal or nodal (as defined in 11.2.1), where no more than 2 lesions are from the same organ and no more than 2 malignant nodal lesions are selected.

Note: If fewer than 5 target lesions and target lymph nodes are identified (as there often will be), there is no reason to perform additional studies beyond those specified in the protocol to discover new lesions.

- Target lesions and target lymph nodes should be selected on the basis of their size, be representative of all involved sites of disease, but in addition should be those that lend themselves to reproducible repeated measurements. It may be the case that, on occasion, the largest lesion (or malignant lymph node) does not lend itself to reproducible measurements in which circumstance the next largest lesion (or malignant lymph node) which can be measured reproducibly should be selected.
- **Baseline Sum of Dimensions (BSD):** A sum of the longest diameter for all target lesions plus the sum of the short axis of all the target lymph nodes will be calculated and reported as the baseline sum of dimensions (BSD). The BSD will be used as reference to further characterize any objective tumor response in the measurable dimension of the disease.
- **Post-Baseline Sum of the Dimensions (PBSD):** A sum of the longest diameter for all target lesions plus the sum of the short axis of all the target lymph nodes will be calculated and reported as the post-baseline sum of dimensions (PBSD). If the radiologist is able to provide an actual measure for the target lesion (or target lymph node), that should be recorded, even if it is below 0.5 cm. If the target lesion (or target lymph node) is believed to be present and is faintly seen but too small to measure, a default value of 0.5 cm should be assigned. If it is the opinion of the radiologist that the target lesion or target lymph node has likely disappeared, the measurement should be recorded as 0 cm.
- **The minimum sum of the dimensions (MSD)** is the minimum of the BSD and the PBSD.

11.4.2 Non-Target Lesions & Non-Target Lymph Nodes

Non-measurable sites of disease (Section 11.2.2) are classified as non- target lesions or non-target lymph nodes and should also be recorded at baseline. These lesions and lymph nodes should be followed in accord with Section 11.4.3.3.

11.4.3 Response Criteria

11.4.3.1 Measurement

All target lesions and target lymph nodes followed by CT/MRI/PET-CT/Chest X-ray must be measured on re-evaluation. Specifically, a change in objective status to either a PR or CR cannot be done without re-measuring target lesions and target lymph nodes.

Note: Non-target lesions and non-target lymph nodes should be evaluated at each assessment, especially in the case of first response or confirmation of response. In selected circumstances, certain non-target organs may be evaluated less frequently. For example, bone scans may need to be repeated only when complete response is identified in target disease or when progression in bone is suspected.

11.4.3.2 Evaluation of Target Lesions

- Complete Response (CR): All of the following must be true:
  a. Disappearance of all target lesions.
  b. Each target lymph node must have reduction in short axis to <1.0 cm.
- Partial Response (PR): At least a 30% decrease in PBSD (sum of the longest diameter for all target lesions plus the sum of the short axis of all the target lymph nodes at current evaluation) taking as reference the BSD (see Section 11.4.1).
- Progression (PD): At least one of the following must be true:
  a. At least one new malignant lesion, which also includes any lymph node that was normal at baseline (<1.0 cm short axis) and increased to ≥1.0 cm short axis during follow-up.
  b. At least a 20% increase in PBSD (sum of the longest diameter for all target lesions plus the sum of the short axis of all the target lymph nodes at current evaluation) taking as reference the MSD (Section **11.4.1**). In addition, the PBSD must also demonstrate an absolute increase of at least 0.5 cm from the MSD.
  c. See Section 11.3.2 for details in regards to the requirements for PD via FDG-PET imaging.
- Stable Disease (SD): Neither sufficient shrinkage to qualify for PR, nor sufficient increase to qualify for PD taking as reference the MSD.

11.4.3.3 Evaluation of Non-Target Lesions & Non-target Lymph Nodes

- Complete Response (CR): All of the following must be true:

a. Disappearance of all non-target lesions.

b. Each non-target lymph node must have a reduction in short axis to <1.0 cm.

- Non-CR/Non-PD: Persistence of one or more non-target lesions or non-target lymph nodes.
- Progression (PD): At least one of the following must be true:

a. At least one new malignant lesion, which also includes any lymph node that was normal at baseline (<1.0 cm short axis) and increased to ≥1.0 cm short axis during follow-up.

b. Unequivocal progression of existing non-target lesions and non-target lymph nodes. (NOTE: Unequivocal progression should not normally trump target lesion and target lymph node status. It must be representative of overall disease status change.)

c. See Section 11.3.2 for details in regards to the requirements for PD via FDG-PET imaging.

11.4.4 Overall Objective Status

The overall objective status for an evaluation is determined by combining the patient's status on target lesions, target lymph nodes, non-target lesions, non-target lymph nodes, and new disease as defined in the following tables:

For Patients with Measurable Disease

Target Lesions & Target Lymph Nodes	Non-Target Lesions & Non-Target Lymph Nodes	New Sites of Disease	Overall Objective Status
CR	CR	No	CR
CR	Non-CR/Non-PD	No	PR
PR	CR Non-CR/Non-PD	No	PR
CR/PR	Not All Evaluated*	No	PR**
SD	CR Non-CR/Non-PD Not All Evaluated*	No	SD
Not all Evaluated	CR Non-CR/Non-PD Not All Evaluated*	No	Not Evaluated (NE)
PD	Unequivocal PD CR Non-CR/Non-PD Not All Evaluated*	Yes or No	PD

Target Lesions & Target Lymph Nodes	Non-Target Lesions & Non-Target Lymph Nodes	New Sites of Disease	Overall Objective Status
CR/PR/SD/PD/Not all Evaluated	Unequivocal PD	Yes or No	PD
CR/PR/SD/PD/Not all Evaluated	CR Non-CR/Non-PD Not All Evaluated*	Yes	PD

* See Section 11.4.3.1

11.4.5 Symptomatic Deterioration:

Patients with global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time, and not either related to study treatment or other medical conditions, should be reported as PD due to "symptomatic deterioration." Every effort should be made to document the objective progression even after discontinuation of treatment due to symptomatic deterioration.

12. TREATMENT/FOLLOW–UP DECISION AT EVALUATION OF PATIENT

12.1 No disease progression or Unacceptable Adverse Events

Patients who have not had disease progression and have not developed unacceptable adverse events will be eligible to continue treatment at their current dose level until disease progression, unacceptable adverse events, or refusal.

12.2 No disease Progression, Unacceptable Adverse Events

Those patients who have not had disease progression, but have experienced unacceptable adverse events may be eligible to continue treatment at a lower dose (see Section 8.0).

12.3 Discontinuation of Protocol Therapy

Reasons why the protocol therapy may be discontinued:

- Tumor progression
- Request by the patient to withdraw
- Unacceptable adverse events
- Inter-current illness
- Administration of alternative treatment

Patients who discontinue treatment for any of the above reasons will proceed to event monitoring. Patients will be followed yearly for a maximum of 5 years from study registration. Once a patient has discontinued study treatment, future therapy is at the discretion of the treating physician.

12.4 Refuse to begin protocol treatment following registration

If an eligible patient refuses to begin treatment following registration (and is classified as a cancel by the Research Base), all on-study materials and the End of Active Treatment/Cancel Notification Form must be submitted. For those patients who also consent to the correlative studies, research biospecimens are not to be submitted. No further data submission is necessary. Future therapy is at the discretion of the treating physician.

12.5 Ineligible prior to the start of protocol treatment after registration

If a patient is determined not to have satisfied each and every eligibility criteria for study entry after registration but prior to the start of protocol treatment, on-study materials must be submitted. For those patients who also consent to the correlative studies, research biospecimens are not to be submitted. Bio-specimens should not be submitted. No further data submission is necessary. Future therapy is at the discretion of the treating physician.

12.6 Ineligible after the start of protocol treatment

If a patient is determined not to have satisfied each and every eligibility criteria but has received some study treatment and is having some clinical benefit, the patient may be able to continue study treatment per protocol after consultation with the study chair.

If the patient does continue on study treatment, study forms and bio-specimens should be submitted using the same schedule as eligible patients.

If the patient does not continue study treatment, complete the End of Active Treatment Form. No further data submission is necessary. Future therapy is at the discretion of the treating physician.

13. ADVERSE EVENT REPORTING REQUIREMENTS

Summary of SAE Reporting for this study
(please read entire section for specific instructions):

WHO:	WHAT form:	WHERE to send:
All sites	Pregnancy Reporting [redacted]	Mayo Sites – complete and attach to MCCC Electronic SAE Reporting Form [redacted] Will automatically be sent to [redacted] Non Mayo sites – complete and forward to [redacted]
Mayo Clinic Sites	MedWatch 3500A: [redacted] AND attach to Mayo Clinic Cancer Center SAE Reporting Form: [redacted]	Will automatically be sent to [redacted]
Non-Mayo Clinic Sites	[redacted]	Send to: [redacted]

13.1 General

Adverse event collection and reporting is a routine part of every clinical trial. This study will use the descriptions and grading scales found in the NCI Common Terminology Criteria for Adverse Events current version (CTCAE v5.0) that is available at [redacted]

Information on all adverse events, whether reported by the participant, directly observed, or detected by physical examination, laboratory test or other means, will be collected, recorded, followed and reported as described in the following sections.

Adverse events experienced by participants will be collected and reported from initiation of study medication, throughout the study, and within 30 days of the last dose of study medication. Participants who experience an ongoing adverse event related to a study procedure and/or study medication beyond 30 days will continue to be contacted by a member of the study team until the event is resolved, stabilized, or determined to be irreversible by the participating investigator.

Participants should be instructed to report any serious post-study event(s) that might reasonably be related to participation in this study. The investigator should notify the IRB and any other applicable regulatory agency of any unanticipated death or adverse event occurring after a participant has discontinued or terminated study participation that may reasonably be related to the study.

13.2 Definitions

13.2.1 Adverse Event (AE)

An adverse event is any undesirable sign, symptom or medical condition or experience that develops or worsens in severity after starting the first dose of study treatment or any procedure specified in the protocol, even if the event is not considered to be related to the study.

Abnormal laboratory values or diagnostic test results constitute adverse events only if they induce clinical signs or symptoms or require treatment or further diagnostic tests.

13.2.2 Serious adverse event (SAE)

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

- is fatal;
- is life-threatening;
- requires or prolongs inpatient hospitalization for ≥24 hours;
- results in persistent or significant disability/incapacity to conduct normal life functions;
- constitutes a congenital anomaly or birth defect; or
- jeopardizes the participant and requires medical or surgical intervention to prevent one of the outcomes listed above;
- Important Medical Events (IME) that may not result in death, be life threatening, or require hospitalization may be considered serious when, based upon medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

Events **not** considered to be serious adverse events are hospitalizations for:

- routine treatment or monitoring of the studied indication, not associated with any deterioration in condition, or for elective procedures
- elective or pre-planned treatment for a pre-existing condition that did not worsen, or that is required per protocol

- emergency outpatient treatment for an event not fulfilling the serious criteria outlined above and not resulting in inpatient admission
- respite care
- Death due to disease progression unless attributable to the study drug(s)
- A hospitalization due to an expected adverse event (e.g., hospitalization due to expected febrile neutropenia).

13.2.3 Expectedness

- Expected: Expected adverse events are those that have been previously identified as resulting from administration of the agent. For the purposes of this study, an adverse event is considered expected when it appears in the current adverse event list, the Investigator's Brochure, the package insert or is included in the informed consent document as a potential risk.
- Unexpected: An adverse event is considered unexpected when it varies in nature, intensity or frequency from information provided in the current adverse event list, the Investigator's Brochure, the package insert or when it is not included in the informed consent document as a potential risk

13.2.4 Attribution

Attribution is the relationship between an adverse event or serious adverse event and the study treatment. Attribution will be assigned as follows:

- Definite – The AE is clearly related to the study treatment.
- Probable – The AE is likely related to the study treatment.
- Possible – The AE may be related to the study treatment.
- Unlikely - The AE is doubtfully related to the study treatment.
- Unrelated - The AE is clearly NOT related to the study treatment.

13.3 Reporting Procedures

13.3.1 General

All adverse events will be captured on the appropriate study-specific case report forms (CRFs).

13.3.2 Serious Adverse Events

All serious adverse events, regardless of causality to study drug, will be reported to the Principal Investigator and/or the Study Coordinator at each institution, and also to the Coordinating Center.

All serious adverse events must be reported to the Coordinating Center within 1 business day after the investigator becomes aware of the event. Events should be reported using a MedWatch form (3500A) as available on the FDA website (see link below).

Follow-up information must also be reported within 1 business day of receipt of the information by the investigator.

The Coordinating Center will disseminate information regarding serious adverse events to the participating sites within 5 days of review of the information by the Protocol Chair (or her designee in the event of extended absence) only in the case that the event(s) is believed to be related (i.e., possibly, probably, or definitely) to the study medication. The Coordinating Center will be responsible for reporting of events to the FDA and supporters, as appropriate (outlined below).

13.3.3 Institutional Review Board

All adverse events and serious adverse events will be reported to the IRB per current institutional standards. If an adverse event requires modification of the informed consent, these modifications will be provided to the IRB with the report of the adverse event. If an adverse event requires modification to the study protocol, these modifications will be provided to the IRB as soon as is possible.

13.3.4 Food and Drug Administration (FDA)

In this trial, unexpected adverse events believed to be definitely, probably, or possibly related to the medications will be reported to the Food and Drug Administration via MedWatch. The Coordinating Center will be responsible for correspondence regarding adverse events with the FDA for all participating sites. Sites will be instructed the method by which to report events to the Coordinating Center and per what forms (e.g., mandatory MedWatch 3500/3500A forms available at:
[redacted]

14. REGULATORY CONSIDERATIONS

14.1 Protocol Review and Amendments

Unless otherwise specified, each participating institution must obtain its own IRB approval. It is expected that the IRB will have the proper representation and function in accordance with federally mandated regulations. The IRB should approve the consent form and protocol.

Information regarding study conduct and progress will be reported to the Institutional Review Board (IRB) per the current institutional standards of each participating center.

Any changes to the protocol will be made in the form of an amendment and must be approved by the IRB of each institution prior to implementation.

The Protocol Chair (or his designee) is responsible for the coordination and development of all protocol amendments, and will disseminate this information to the participating centers.

14.2 Informed Consent

The investigator (or his/her designee) will explain to each subject 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 will be informed that participation in the study is voluntary, that s/he may withdraw from the study at any time, and that

withdrawal of consent will not affect her subsequent medical treatment or relationship with the treating physician(s) or institution. The informed consent will be given by means of a standard written statement, written in non-technical language, which will be IRB approved. The subject should read and consider the statement before signing and dating it, and will be given a copy of the document. No subject will enter the study or have study-specific procedures done before his/her informed consent has been obtained.

In accordance with the Health Information Portability and Accountability Act (HIPAA), the written informed consent document (or a separate document to be given in conjunction with the consent document) will include a subject authorization to release medical information to the study sponsor and supporting agencies and/or allow these bodies, a regulatory authority, or Institutional Review Board access to subjects' medical information that includes all hospital records relevant to the study, including subjects' medical history.

14.3 Ethics and GCP

This study will be carried out in compliance with the protocol and Good Clinical Practice, as described in:

1. ICH Harmonized Tripartite Guidelines for Good Clinical Practice 1996.
2. US 21 Code of Federal Regulations dealing with clinical studies (including parts 50 and 56 concerning informed consent and IRB regulations).
3. Declaration of Helsinki, concerning medical research in humans (Recommendations Guiding Physicians in Biomedical Research Involving Human Subjects, Helsinki 1964, amended Tokyo 1975, Venice 1983, Hong Kong 1989, Somerset West 1996).

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.

14.4 Compliance with Trial Registration and Results Posting Requirements

Under the terms of the Food and Drug Administration Modernization Act (FDAMA) and the Food and Drug Administration Amendments Act (FDAAA), the sponsor-investigator of the trial is solely responsible for determining whether the trial and its results are subject to the requirements for submission to the Clinical Trials Data Bank, [redacted] Information posted will allow subjects to identify potentially appropriate trials for their disease conditions and pursue participation by calling a central contact number for further information on appropriate trial locations and study site contact information.

15. MULTI-CENTER GUIDELINES

15.1 Study Documentation

Each participating site is responsible for submitting copies of all relevant regulatory documentation to the Coordinating Center. The required documents include, but are not limited to the following: local IRB approvals (i.e., protocol, consent form, amendments, patient brochures and recruitment material, etc.), IRB membership rosters, summary of unanticipated problems or protocol deviations, and documentation of expertise of the investigators. The Coordinating Center will provide each participating site with a

comprehensive list of the necessary documents. It is the responsibility of the participating sites to maintain copies of all documentation submitted to the Coordinating Center.

The requirements for data management, submissions, and monitoring are outlined in the Data Safety Monitoring Plan [provided separately].

15.2 Records Retention

Following closure of the study, each participating center will maintain a copy of all site study records in a safe and secure location. The Coordinating Center will inform the investigator at each site at such time that the records may be destroyed.

15.3 Publication

It is understood that any manuscript or releases resulting from the collaborative research must be approved by the Protocol Chair and will be circulated to applicable participating sites/investigators prior to submission for publication or presentation.

Additionally, any publication of study data and results must conform to the publications policy as stated the Translational Breast Cancer Research Consortium's (TBCRC) "Policies and Procedures".

16. STATISTICAL CONSIDERATIONS

An archived FFPE tumor tissue from a locally advanced or metastatic site or from the primary tumor specimen if a locally advanced or metastatic site is not available will be submitted to the Mayo Clinic CAP/CLIA-certified Anatomic Pathology Laboratory for ERβ testing. If the specimen is found to be ERβ+, the patient may registered to the study.

If the specimen submitted was from a tissue-based biopsy of a local recurrence or metastatic site taken at most 12 months prior to Pre-Registration, a new biospy is not required prior to the start of treatment. These patients will be included in the ERβ+ cohort for analysis purposes.

If the specimen submitted is not from a tissue-based biopsy of a local recurrence or metastatic site taken at most 12 months prior to Pre-Registration, a new biopsy of a local recurrence or metastatic site must be performed prior to the start of treatment and the specimen submitted for central laboratory testing and correlative research aims. The results of this ERβ testing does not impact whether the patient remains on protocol treatment. It does however impact how the primary and secondary endpoints are accessed. If this new tissue specimen is found to be ERβ+, the patient will be included in the ERβ+ cohort for analysis purposes. If this new tissue specimen is found to be ERβ-, the patient will be included in the ERβ- cohort for analysis purposes.

All eligible patients who begin treatment will be included in the analyses of the primary and secondary clinical endpoints.

16.1 Study Design

Primary endpoint of this trial: 6 month clinical benefit rate

A patient is said to have derived clinical benefit rate at the 6 month time point if the patient's disease meets the RECIST criteria for complete response (CR), partial response (PR), or stable disease (SD) for >6 months following initiation of treatment. The 6 month clinical benefit rate is the percentage of patients who are found to meet the criteria for clinical benefit at least 6 months among all the patients who have started estradiol treatment.

ERβ+ cohort

A single-stage, phase II trial will be conducted to assess independently the 6 month clinical benefit rate (CBR6m) among patients with moderate to strong ERβ expressing metastatic TNBC treated with estradiol.

With a sample size of 38 patients with moderate to strong ERβ expressing TNBC, a one-sided α=0.10 test of binomial proportions would have a 90% chance of rejecting the null hypothesis that the CBR6m is ≤5% when the true CBR6m is ≥20% in this patient population.

ERβ- cohort

As the number of patients with discordant ERβ findings are expected to be small, a 90% exact binomial confidence interval will be constructed for the proportion of patients who were found to have no to weak ERβ expressing metastatic TNBC and who derived clinical benefit rate at the 6 month time point.

For patients with a history of moderate to strong ERβ expressing TNBC who are registered onto this study, it is unknown what percentage of these patients will not have moderate to strong ERβ expression found in their pre-registration biopsy specimen. After every 5th patient is registered to the study (up to 30 patients), we will examine the number of the patients who do not have moderate to strong ERβ expression found in their pre-registration biopsy specimen as follows:

If ≥2 of the first 5 patients registered,

≥2 of the first 10 patients registered,

≥2 of the first 15 patients registered,

≥3of the first 20 patients registered,

≥3 of the first 25 patients registered,

or ≥4 of the first 30 patients registered

are found to have no or weak ERβ expression, the trial will temporarily close to assess whether the screening/pre-registration process should be modified to ensure accrual only for those patients with moderate to strong ERβ expressing metastatic TNBC.

Based on the experience of TBCRC 011 enrolling patients with histologically confirmed ERα negative, PgR-negative MBC who consented for AR testing, we expect 20% of patients to have moderate to strong ERβ expression found in the archived locally recurrent or metastatic TNBC specimen they submit in the prescreening phase. Also, we anticipate that 5-10% of these patients may be found to have no or weak ERβ expression found in their pre-registration biopsy specimen. Thus, we anticipate pre-screening as many as 210 patients over 24-30 months to enroll 38 patients whose pre-registration biopsy specimen is found to have moderate to strong ERβ expression.

16.1.1 Interim analysis for futility and toxicity

An interim analysis for futility among the patients whose pre-registration biopsy specimen is found to have moderate to strong ERβ expression will be carried out based on Simon Two-stage Optimum Phase II trial design for testing the null hypothesis that the true CBR6m is at most 5% against the alternative hypothesis that the true CBR6m is at least 20% with the significance level set at 0.10 and the power at 0.90. Specifically, after the first 19 eligible patients have enrolled and either completed 6 months of estradiol or discontinued treatment (for any reason) prior to completing 6 months of treatment, an interim analysis will be performed. If at most 1 patient has remained on treatment more than 6 months, then the trial will be closed to enrollment due to futility. The probability of early termination is 0.755.

Safety Stopping Rule for both patient cohorts combined (as the ERβ results for those whose submit a second biopsy sample will be delayed)

16.1.2

If treatment intolerability is encountered, the trial will temporarily suspend enrollment so that the study team can review the toxicity data and develop a recommendation to present to MCCC DSMB and IRB for approval.

We will temporarily halt enrollment:

- If 2 or more of the first 6 patients enrolled, develop a Grade 3+ hypercalcemia, thrombosis, or pleural effusions, or any Grade 4+ event considered to be at least possibly related to treatment during the first cycle of treatment.
- If at any point in the trial after the first 6 patients have been enrolled, the percentage of patients who develop a Grade 3+ hypercalcemia, thrombosis, or pleural effusion, or any Grade 4+ event considered to be at least possibly related to treatment at any point in their treatment exceeds 30%.

16.2 Analysis of Secondary Endpoints for each patient cohort independently

16.2.1 Adverse Events

An adverse event (AE) is any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the

use of a medical treatment or procedure that may or may not be considered related to the medical treatment or procedure. All Grade 2, 3, 4 or 5 adverse events will be documented and assigned an attribute by treating clinician as to its relationship to treatment.

For a given AE, the proportion of patients who report developing a Grade 2-5 of this AE will be determined.

The number of dose reductions per patient and the reasons for the dose reduction will be summarized.

16.2.2 Tumor Response Rate among those patients with measurable disease

The tumor response rate is defined as the 100% time the number of patients with a CR or PR (as defined by the RECIST criteria) on 2 consecutive evaluations at least 8 weeks apart divided by the total number of eligible patients who began study treatment. A 90% binomial confidence interval will be constructed for the true response rate.

16.2.3 Progression-free survival distribution

Progression-free survival time is defined as the time from registration to the first of the following events: local, regional, or distant recurrence, second primary disease or death due to any cause. The distribution of PFS times will be estimated using the method of Kaplan-Meier.

16.2.4 Overall survival distribution

Overall survival time is defined as the time from registration to death due to any cause. The distribution of survival times will be estimated using the method of Kaplan-Meier.

16.2.5 Evaluation of changes in phospho-ERβ, cystatins 1, 2, 4 and 5, phospho-Smad2/3 and Ki-67.

Patients will undergo tumor biopsies prior to the start of treatment and at completion of cycle 1 treatment. These specimens will be undergo IHC staining with the following antibodies: phospho-ERβ, cystatins 1, 2, 4 and 5, phospho-Smad2/3 and Ki-67. These data will be used to examine the hypothesis that estradiol will activate ERβ resulting in (1) downregulation of Ki-67, (2) reduction in phospho-Smad2/3 (3) increase in phosphorylation (activation) of ERβ and finally (4) increase in expression levels of cystatins 1, 2, 4, and 5.

For each of these biomarkers, a times series plot will be constructed so that an individual patient's data will be represented using the same color for each of the five graph. These graphs will be visually inspected for trends within each of the graphs (variation between individuals) as well as across the five graphs (profile of biomarker changes within an individual).

16.2.6 Changes in serum cystatin levels in response to treatment

Blood specimens will be acquired prior to the start of treatment and at completion of cycle 1 treatment. Serum will undergo ELISA testing to obtain cystatins 1, 2, 4 and 5 levels. These data will be used to examine the hypothesis that the clinical benefit of estradiol will be associated with an increased in cystatins 1, 2, 4 and 5.

For each of the cystatins, the percent change in its level following one cycle of treatment will examined using signed rank tests and the difference in the percent change in its level following one cycle of treatment between those patients who derived clinical benefit and those who did not will be examined using a two sample Wilcoxon rank sum test.

16.3 Monitoring

The study chair and the faculty statistician will review the trial data every 3 months to identify accrual, toxicity, and endpoint problems that might be developing. The faculty and secondary statistician will prepare a report containing accrual, adverse events, and efficacy data which will be submitted to the Data and Safety Monitoring Board on a semi-annual basis.

16.4 Other Considerations

Adverse events, patterns of treatment failure observed in this study and scientific discoveries or changes in standard care will be taken into account in any decision to terminate this trial earlier than anticipated.

16.5 Inclusion of Women and Minorities

This study will be available to all eligible patients regardless of race or ethnic group. The expected number of patients per racial/ethnicity categories are presented in the following table. The sample size for this trial was not increased in order to provide additional power for analyses by race or ethnicity.

NOTE: We have excluded men from enrollment in this trial for two reasons:
1) Breast cancer is not common in males so only one or two patients are likely to be enrolled; and
2) Because this trial is small, the addition of one or two male patients may confound the results without providing meaningful data.

Accrual Targets			
	Sex/Gender		
Ethnic Category	**Females**	**Males**	**Total**
Hispanic or Latino	21		21
Not Hispanic or Latino	189		189
Ethnic Category: Total of all subjects	**210**	**0**	**210**
Racial Category			
American Indian or Alaskan Native			
Asian	8		8
Black or African American	8		8
Native Hawaiian or other Pacific Islander			
White	194		194
Racial Category: Total of all subjects	**210**	**0**	**210**

Ethnic Categories:

Hispanic or Latino – a person of Cuban, Mexican, Puerto Rican, South or Central American, or other Spanish culture or origin, regardless of race. The term "Spanish origin" can also be used in addition to "Hispanic or Latino."

Not Hispanic or Latino

Racial Categories:

American Indian or Alaskan Native – a person having origins in any of the original peoples of North, Central, or South America, and who maintains tribal affiliations or community attachment.

Asian – a person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian subcontinent including, for example, Cambodia, China, India, Japan, Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam. (Note: Individuals from the Philippine Islands have been recorded as Pacific Islanders in previous data collection strategies.)

Black or African American – a person having origins in any of the black racial groups of Africa.

Native Hawaiian or other Pacific Islander – a person having origins in any of the original peoples of Hawaii, Guam, Samoa, or other Pacific Islands.

White – a person having origins in any of the original peoples of Europe, the Middle East, or North Africa.

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APPENDICES

Appendix A: ECOG Performance Status Scale

Appendix B: Study Drug Diary

APPENDIX A: ECOG Performance Status Scale

ECOG PERFORMANCE STATUS*	
Grade	**ECOG**
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all selfcare but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited selfcare, confined to bed or chair more than 50% of waking hours.
4	Completely disabled. Cannot carry on any selfcare. Totally confined to bed or chair.
5	Dead

*As published in Am. J. Clin. Oncol.:
Oken, M.M., Creech, R.H., Tormey, D.C., Horton, J., Davis, T.E., McFadden, E.T., Carbone, P.P.: Toxicity And Response Criteria Of The Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649-655, 1982.

The ECOG Performance Status is in the public domain therefore available for public use. To duplicate the scale, please cite the reference above and credit the Eastern Cooperative Oncology Group, Robert Comis M.D., Group Chair.

[redacted]

APPENDIX B: Study Drug Diary

PLEASE NOTE: Study Drug Diary for patient care use is posted as a standalone document. Remember to submit to your local IRB before using with a patient.