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TITLE: Targeting Androgen Receptor in Breast Cancer: Enzalutamide as a Novel Breast Cancer Therapeutic

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14. ABSTRACT In breast cancer the androgen receptor (AR) is more widely expressed than estrogen receptor alpha (ER) or the progesterone receptor (PR), suggesting a potential role for AR in BC. To explore the function of AR in models of the three main subtypes of breast cancer (ER positive, ER negative and Her2+), we are using a new-generation AR inhibitor, enzalutamide (Enza), which impairs nuclear localization of AR. Our research seeks to determine whether Enza will be effective in breast cancer and utilize preclinical models to determine if and how it should be combined with standard treatments with the primary objective being to guide future clinical trials. In Dec 2015 Drs. Elias and Richer, demonstrate synergy between Enza and Tamoxifen or Fulvestrant <i>in vitro</i> and results of a Phase 1 study (NCT01597193) on pharmacokinetics and safety of Enza plus Fulvestrant in women with advanced ER+ disease. Regarding TNBC, we reported that AR is anti-apoptotic and facilitates anchorage independent growth and Enza decreased tumor viability <i>in vivo</i> . AR regulates secreted factors that support cancer stem cells and Enza significantly decreased tumor initiating capacity of TNBC <i>in vivo</i> . Enza administered to mice with chemotherapy greatly decreased TNBC tumor burden. These findings have guided future clinical trials with Enza in breast cancer. We report on the two trials in ER+ breast cancer that are now completed.					
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Title: Targeting Androgen Receptor in Breast Cancer: Enzalutamide as a Novel Breast Cancer Therapeutic

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Contracting Organization: University of Colorado Anschutz Medical Campus

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Introduction

The central thesis of this grant is to understand the role of AR signaling in breast cancer subtypes and understand how to best use an inhibitor of AR signaling, enzalutamide (enza), as a therapeutic agent in breast cancer. With the recognition that AR is expressed in all subtypes of breast cancer, that overexpression is frequently associated with relative resistance to therapy (both anti-estrogen and chemotherapy) (work of our group and others), and with the advent of increasingly potent AR signaling inhibitors in prostate cancer, the area of anti-AR therapeutics in breast cancer is one of the most active worldwide. The preclinical portion of this grant serves to understand mechanism of action of AR signaling inhibition alone or in combination with other targeted agents in ER+, Her2+, or TNBC in preclinical models, and then perform biomarker analysis in human tissues obtained before, during and after treatment with enzalutamide. The clinical portion of this grant serves to obtain these tissues in concert with the overall clinical development of enzalutamide in the subtypes of breast cancer.

Keywords

Breast cancer (BC) subtypes; androgen receptor (AR); preclinical modeling; enzalutamide; AR inhibition; resistance mechanisms; predictive biomarkers; targeted therapy.

Overall Project Summary

The objective of Stage I of this proposal was to rapidly generate preclinical data in the laboratory of Jennifer Richer, Ph.D., testing the anti-androgen enzalutamide alone or in combination with standard of care therapeutics in different subtypes of BC to help guide the clinical trials described in **Stage II** (PI clinical partner Dr. Anthony Elias) and steer the rational design and focus on patients most likely to benefit from enzalutamide alone or in combination with currently used therapeutics. Below we describe for each task in the official statement of work the major activities; specific objectives; significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or other achievements. We include a discussion of stated goals not met or tasks not fully completed. We include pertinent data and graphs in sufficient detail to explain significant results achieved. Detailed description of the methodology used is provided in the methods section of two manuscripts in the appendix. The first manuscript was published in January of 2014 and was submitted with the first annual progress report. The second manuscript arising from this work investigated AR in the non-LAR subtype of TNBC was published in 2015 (Barton VN et al MOL CA

THER 2015). A third primary manuscript on AR in ER+ breast cancer came out in 2016 (D'Amato NC *MOL CA RES* 2016). In July 2017 we published an additional two primary manuscripts, one on AR in HER2+ breast cancer and synergy between enzalutamide and everolimus or trastuzumab. Everolimus was found in clinical trials to be very effective in ER+ breast cancer, but less so in HER2+ and TNBC. Our studies show that everolimus increases the amount of AR and AR activity and therefore combining the anti-androgen enzalutamide with everolimus gives a synergistic effect over either drug alone (Gordon MA et al *MOL CA THER* 2017). The next study demonstrated that AR is anti-apoptotic, supports anchorage independent growth and androgens expand a cancer stem cell-like population in TNBC (Barton VN. And Christenson JL, *CAN RES* 2017). We reported that enzalutamide given either simultaneous with or sequential to chemotherapy and found that simultaneous treatment was more effective at preventing recurrence after cessation of chemotherapy, a very important finding for future clinical trial design. A sixth manuscript with a colleague in my department in which we reported on a collaboration that determined that AR supported tumor progression in a preclinical model of obesity after the loss of ovarian function (Wellberg EA, et al *HORMONES AND CANCER*, 2017). While no DOD funds from the Richer lab were used for that study, we did use enzalutamide to show the AR involvement in that study and it is very pertinent to topic of this grant), so we mention it here. We also published 4 review articles on the topic of AR in breast cancer: The publications are all listed in the products section. Recent publications focused on genes regulated by AR in AR+ TNBC PDX and how AR targeting works with CDK4/6 inhibitors. Three recent publications were on AR regulated secreted factors in breast cancer, AR in *ESR1* mutated metastatic breast cancer and the latest on the mutual exclusivity between *ESR1* mutations and *TP53* mutations in metastatic breast cancer. The manuscript on the results of the 16-1001 clinical trial of fulvestrant plus enzalutamide in metastatic ER+ breast cancer was accepted and is published as of May 2023 (Elias AE et al *NPJ Breast cancer* 2023 PMID: 37210417). Our last goal is to submit for review the manuscript on the neoadjuvant 16-1042 trial with one arm being fulvestrant alone and the other fulvestrant plus enzalutamide.

Key Research Accomplishments

Preclinical Aim 1. To test enzalutamide (enza) in combination with currently approved therapies for breast cancer (BC) in the various subtypes of BC.

Task 1. Evaluate enzalutamide in combination with anti-estrogen therapy in ER+/AR+ BC lines (MCF7, BCK4) and a ER+/AR+ patient derived xenograft. 100% completed. Results published summarized below and published in D'Amato NC *et al MOL CA RES* 2016. Androgen receptor (AR) is expressed in 90% of estrogen receptor alpha positive (ER+) breast tumors, but its role in tumor growth and progression remains controversial. Use of two anti-androgens that inhibit AR nuclear localization, enzalutamide and MJC13, revealed that AR is required for maximum ER genomic binding. Here, a novel global examination of AR chromatin binding found that estradiol induced AR binding at unique sites compared to dihydrotestosterone (DHT). Estradiol-induced AR binding sites were enriched for estrogen response elements and had significant overlap with ER binding sites. Furthermore, AR inhibition reduced baseline and estradiol-mediated proliferation in multiple ER+/AR+ breast cancer cell lines and synergized with tamoxifen and fulvestrant.

Task 2. Test enzalutamide in tamoxifen resistance models *in vitro*. 100% completed. *In vivo*, enzalutamide significantly reduced viability of tamoxifen-resistant MCF7 xenograft tumors and an ER+/AR+ patient derived model. Enzalutamide also reduced metastatic burden following cardiac injection. Lastly, in a comparison of ER+/AR+ primary tumors versus patient-matched local recurrences or distant metastases, AR expression was often maintained even when ER was reduced or absent. These data provide preclinical evidence that anti-androgens that inhibit AR nuclear localization affect both AR and ER, and are effective in combination with current breast cancer therapies. In addition, single agent efficacy may be possible in tumors resistant to traditional endocrine therapy, since clinical specimens of recurrent disease demonstrate AR expression in tumors with absent or refractory ER. The first therapy of choice to treat an ER+ tumor, at least at the current

time is an anti-estrogen or aromatase inhibitor. We showed that the relative expression of AR to ER protein (percent cells positive) can predict a poor response to tamoxifen and poor overall survival (Cochrane DR et al 2014). We determined that the enzalutamide is efficacious in tamoxifen resistant MCF 7 cells *in vivo*. However, ER+ tumors will likely be treated first with tamoxifen (if the woman is premenopausal) or aromatase inhibitor (AI) if post-menopausal or having recurred while on tamoxifen, then if there is a recurrence of disease, with the ER degrader Fulvestrant. Therefore, we tested for synergy between these two drugs (D'Amato NC *et al* **MOL CA RES** 2016).

Task 3. Test enzalutamide in combination with HER2-directed therapy in ER+ and ER- HER2+ models. (100% complete) published in (Gordon MA *et al* **MOL CA THER** 2017). **Abstract:** The androgen receptor (AR) is widely expressed in breast cancer, and evidence suggests dependence on AR signaling for growth and survival. AR antagonists such as enzalutamide and seviteronel have shown success in preclinical models and clinical trials of prostate cancer and are currently being evaluated in breast cancer. Reciprocal regulation between AR and the HER2/PI3K/mTOR pathway may contribute to resistance to HER2- and mTOR-targeted therapies; thus, dual inhibition of these pathways may synergistically inhibit breast cancer growth. HER2⁺ and triple-negative breast cancer cell lines were treated with AR antagonist plus anti-HER2 mAb trastuzumab or mTOR inhibitor everolimus. Apoptosis, cell proliferation, and drug synergy were measured *in vitro*. Pathway component genes and proteins were measured by qRT-PCR, Western blot, and reverse phase protein array. *In vivo*, HER2⁺ breast cancer xenografts were treated with enzalutamide, everolimus, trastuzumab, and combinations of these drugs. AR antagonists inhibited proliferation of both HER2⁺ and TNBC cell lines.

Task 4. Examine enzalutamide in combination with an mTOR inhibitor (Afinitor/everolimus). (100% complete) published in (Gordon MA *et al* **MOL CA THER** 2017). Combining AR antagonist and either everolimus or trastuzumab resulted in synergistic inhibition of proliferation. Dihydrotestosterone caused increased phosphorylation of HER2 and/or HER3 that was attenuated by AR inhibition. Everolimus caused an increase in total AR, phosphorylation of HER2 and/or HER3, and these effects were abrogated by enzalutamide. Growth of trastuzumab-resistant HER2⁺ xenograft tumors was inhibited by enzalutamide and combining enzalutamide with everolimus decreased tumor viability more than either single agent. AR antagonists synergize with FDA-approved breast cancer therapies such as everolimus and trastuzumab through distinct mechanisms. Treatment combinations are effective in trastuzumab-resistant HER2⁺ breast cancer cells *in vivo*.

Task 5. In TNBC cell lines and explants that retain AR, enzalutamide will be evaluated alone and in combination with chemotherapy and everolimus *in vitro* and *in vivo*. (100% completed. Published in Barton VN and Christenson JL *et al* **CANCER RES** 2017). **Abstract:** Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype lacking estrogen and progesterone receptors, and human epidermal growth factor receptor 2 (HER2). While to date there are no approved targeted therapies for TNBC, preclinical and early clinical trials indicate that up to 50% express some degree of positivity for androgen receptors (AR) and are sensitive to AR targeted therapy. However, the function of AR in TNBC and the mechanisms by which AR targeted therapy reduces tumor burden in preclinical and clinical settings are unknown. We hypothesized that AR maintains a cancer stem cell-like (CSC) tumor initiating population and that it serves as an anti-apoptotic factor that facilitates anchorage independence. **Methods** Anchorage independence/anoikis resistance was assessed on poly-Hema coated tissue culture plates used to achieve forced suspension culture and apoptosis was measured with cleaved caspase 3 antibody. AR was inhibited using the AR inhibitor Enzalutamide (Enza) or shRNAs targeting AR. CSC populations were assessed *in vitro* using ultra low attachment plates, CD44/CD24 staining, the ALDEFLUOR assay, and single cell mammosphere formation efficiency (MFE) assays in TNBC cell lines SUM159PT and MDA-MB-453.. *In vivo*, tumor-initiating capacity was assessed using a limiting dilution assay of SUM159PT cells pre-treated with or without Enza. Lastly, the efficacy of combination Enza

and chemotherapy was assessed by caliper measurement and intravital imaging of TNBC xenografts in mice treated with Enza and paclitaxel. **Results** AR transcript ($P<0.05$), protein, and transcriptional activity ($P<0.01$) increased in tumor cells in suspension culture compared to attached conditions. Cells that expressed AR protein resisted detachment-induced apoptosis. The CSC population increased in suspension culture by ALEDfluor staining ($P<0.01$), CD44/CD24 staining ($P<0.001$), and MFE ($P<0.05$). AR inhibition decreased ADLH staining ($P<0.001$), increased CD24 staining ($P<0.05$), and decreased MFE ($P<0.01$). In vivo, pre-treatment with Enza decreased the tumor-initiating capacity of TNBC cells in a limiting dilution assay ($P<0.05$). Enza significantly decreased tumor volume and viability when administered during or after chemotherapy in vivo ($P<0.05$) and simultaneous treatment significantly reduced tumor recurrence. In conclusion, AR supports anchorage independence, maintenance of CSCs, tumor initiation and regrowth following chemotherapy in a TNBC preclinical model. Thus, AR targeted therapies may enhance the efficacy of chemotherapy even when there are few cells positive for AR, perhaps by targeting the CSC-like population that proliferates more slowly and is resistant to chemotherapy.

Preclinical Aim 2. Using samples collected from the xenograft studies, examine if and how the mechanism of action by which enzalutamide works in the various subtypes of breast cancer. (100% complete)

- **Task 1.** Perform IHC on xenograft tumors for AR, ER, Her3, BrdU, FOXA1, PSA, SDF1, Cyr61. Months 12-18. We have performed IHC for all these proteins from xenograft experiments on ER+, HER2+ and TNBC where relevant (see published papers and AR+ TNBC PDX below in Figure 2. We also think the AR regulation of the EGFR ligand amphiregulin is very important (Barton V et al 2015).
- **Task 2.** Make RNA from xenografts, perform RNA sequencing analyze. Months 15-18 (100% completed). We analyzed RNA profiling from ER+ and TNBC cell line xenografts as reported in previous progress reports. Additionally, we performed RNA-seq on ER+PDX (the PT12 PDX grown with E2 plus or minus Enza and reported the analysis in the D'Amato Mol Ca Res 2016 paper. Inhibiting AR with Enza, inhibits many classic E2/ER and AR regulated genes.
- In 2020/2021 we published Christenson JL et al Activity of combined androgen receptor antagonism and cell cycle inhibition in androgen receptor-positive triple-negative breast cancer *MOL CA THER* 2021 June 20(6):1062-107. PMID: 33722849 examining RNA-sequencing data from an AR+ TNBC PDX HCI-009 that increases in size in response to DHT. We identified numerous known AR regulated genes such as prostate specific antigen (PSA), also called KLK3 that is used as a marker of disease burden in prostate cancer and we are comparing this to genes that are downregulated in the presence of the anti- androgen enzalutamide in this and other TNBC PDX. We showed that Although cell cycle cyclin-dependent kinase (CDK) 4/6 inhibitors are approved for treatment of ER-positive (ER^+) breast cancer, they have not proven effective as monotherapy in patients with TNBC. The androgen receptor (AR) has emerged as a therapeutic target in a subset of TNBCs and with significant clinical benefit observed in multiple trials. The purpose of this study was to investigate the preclinical activity of the CDK4/6 inhibitor, abemaciclib, in combination with an agent that targets both androgen biosynthesis and AR activity, seviteronel, using TNBC cell lines expressing high AR, cell line xenografts, and an AR-positive (AR^+), androgen-responsive TNBC patient-derived xenograft (PDX). Single-cell RNA sequencing demonstrated heterogeneity in AR levels, even in a highly AR^+ cell line, and identified cell cycle pathway activation in AR^{High} - versus AR^{Low} -expressing cells. Combination treatment with the cell cycle CDK4/6 inhibitor, abemaciclib, and seviteronel showed synergy in an AR^+ TNBC model compared with each drug alone. Although cell cycle inhibitors are FDA approved for use in ER^+ breast cancer, our studies suggest that they may also be effective in AR^+ TNBC combined with AR-targeted agents.

Preclinical Aim 3. Identify mechanisms of resistance to enzalutamide in triple negative breast cancers to

elucidate pathways that impinge on the AR pathway to potentially target in combination with enzalutamide. (100% complete)

- **Task 1.** Sequence AR+ triple negative cell lines resistant and 3 that are sensitive. Months 18-24
Since the TNBC cell lines that we have studied so far are sensitive to enzalutamide and we have found it to particularly affect growth on soft agar (Barton V et al 2015), we have not performed sequencing of all of these yet because we are still trying to figure out the best conditions and timing. We are also exploring another approach, which is to chronically treat the cells with enza to generate resistant lines. We have taken this approach with the MDA-MB-453 TNBC line which we showed in Cochrane et al 2014 to be very responsive to enza in vitro and in vivo. We now have a resistant line that we did mutational analysis on the resistant line and it does not have the F876L mutation that has been reported to confer resistance to enzalutamide in prostate cancer cells and patient tumors. It is likely that the cell line that we have rendered resistant is resistant via a different mechanism other than this AR mutation.

Summary for this aim:

- No completely resistant TNBC lines. Enza IC50s does not correlate with AR protein.
- No clearly “sensitive” versus “resistant” cell lines. Still looking at PDX. Do have MDA-MB-231 with extremely low AR and they express much more glucocorticoid receptor GR.
- We published that AR regulates TGFβ ligands and receptors: “Feedback Loop Between TGFβ and Androgen Receptor Supports Triple-Negative Breast Cancer Anoikis Resistance. *ENDOCRINOLOGY* 2021 Feb 1;162(2) PMID: 3329492283, by Rosas E. *et al.* AR expression is increased in anchorage-independent cells in TNBC preclinical models. Both AR knockdown and inhibition lead to reduced TNBC invasion in vitro, reduced tumorigenicity, and less recurrence in vivo in preclinical models. Transforming growth factor β (TGFβ) pathway gene signatures also increased during anchorage-independent survival both in vitro and in vivo in preclinical models and in circulating tumor cells (CTCs) from patients during emergence of chemo resistant disease. We hypothesized that a positive loop between AR and TGFβ signaling facilitates TNBC anchorage-independent survival. We find that multiple components of the TGFβ pathway, including TGFβ1 and 3, as well as pathway activity measured by nuclear localization and transcriptional activity of phosphorylated Smad3, are enhanced in anchorage-independent conditions. Further, exogenous TGFβ increased AR protein while TGFβ inhibition decreased AR and TNBC viability, particularly under anchorage-independent culture conditions. ChIP-seq experiments revealed AR binding to TGFB1 and SMAD3 regulatory regions in MDA-MB-453 cells. In clinical datasets, TGFB3 and AR positively correlate and high expression of both genes together corresponded to significantly worse recurrence-free and overall survival in both ER-negative and basal-like breast cancer. Finally, inhibiting both AR and TGFβ decreased cell survival, particularly under anchorage-independent conditions. These findings warrant further investigations into whether combined inhibition of AR and TGFβ pathways might decrease metastatic recurrence rates and mortality from TNBC.

Dr. Richer’s award (W81XWH-13-1-0090) for the preclinical work no longer has funds, but she is in charge of the molecular laboratory correlates/analyses of the serial biopsies research and the PRA Nicole Spoelstra in her lab is completing all immunohistochemistry. Our **two clinical trials were approved by our local IRB (COMIRB 16-1042 and COMIRB 16-1001)** to complete the clinical aims of this grant. **Both trials are now complete as reported in Partnering PI Dr. Elias’s final report.**

Clinical Aim 1: To identify pretreatment molecular characteristics associated with lack of response and/or prolonged PFS (Patient Tissues).

Clinical Aim 2: To determine if a decrease in Ki67 or increase in apoptosis as measured by TUNEL in biopsies taken before treatment as compared to after 2-4 weeks of treatment or other to be determined genes or proteins are associated with lack of response and/or prolonged PFS.

Clinical Aim 3: To determine if changes in molecular determinants between pre-treatment biopsies and tissue at time of disease progression can help identify resistance mechanisms.

Task 1: Serial Biopsy Trial (Elias, Traina, Schwartzberg, Petricoin, Patient advocates, Richer)

- The DOD sponsored serial biopsy trial titled “Exploratory Development of Predictive Biomarkers for Patients with Androgen-Receptor Positive (AR) Breast Cancer (BC) Treated with Enzalutamide (MDV3100); COMIRB 13-1473,” was activated at the University of Colorado site and the West Clinic/University of Tennessee site. It is now closed to accrual at all sites due to the reasons stated below.
- 6 patients with serial biopsies were enrolled.
 - Tissues studies were reported in previous years’ Progress Report.
 - Please see the Annual Report for Award Number W81XWH-13-1-0090 for all laboratory work done on the tissues and for all preclinical work.
- Accrual to this trial was not completed due to the limitation that this biopsy trial was a companion to therapeutic trials of enzalutamide in breast cancer sponsored by Medivation and Astellas. Because those therapeutic trials were opened to large numbers of institutions that were not part of our DOD grant, and were completed very quickly, our accrual was limited.
- On the other hand, the clinical development of enzalutamide was enhanced, making possible the forward-thinking investigator-sponsored trials as outlined for Years 3-5 in our grant.
- The trials in Clinical Aims 4 and 5 did include serial biopsies, and allowed us to complete the Clinical Aims 1-3 albeit not with patients treated with single agent enzalutamide.
- Please see the Annual Report for Award Number W81XWH-13-1-0090 for all laboratory work done on the tissues and for all preclinical work.
- Heterogeneous patients with respect to ER status, prior treatment.
- ER/AR IHC from archived tissue (obtained from primary tumor) often dramatically different from the immediate pre-treatment biopsy.
- Two patients with ER+ disease had mutated ESR1 in the pretreatment biopsy
- Using RPPA methodology to examine phosphoproteins, pre- vs post-enzalutamide tissues frequently demonstrated downregulation of various growth factor pathways.

Clinical Aim 4: To determine if enza can overcome *de novo* resistance to exemestane in postmenopausal women with T2 or larger ER+ BC treated preoperatively: protocol 16-1042: a randomized phase II trial of fulvestrant with or without enzalutamide as preoperative treatment for women with ER+/AR+/Her2-breast cancer was activated for accrual on 08/30/2017 at the University of Colorado. See details about the design and approval for this trial and the one in clinical Aim 5 for metastatic disease in Dr. Elias’s annual report. This trial was activated at MSKCC upon resolution of Astellas contracts on 7/2/18. It was also activated at University of Tennessee. Stage I accrual was completed on 03/02/2021, with 22 evaluable subjects enrolled on the experimental (combination) arm. More than 4 patients on the combination arm achieved a PEPI score = 0, therefore an additional 16 patients were enrolled in stage 2. Stage 2 accrual was completed on 10/14/2021. 69 subjects signed consent, 61 of which were treated one of which withdrew prior to starting treatment. There were also three screen failures and four patients who withdrew prior to enrollment. All patients have now completed treatment/surgery and the last surgical resection was completed on 02/17/2022. 45 subjects have evaluable tissue at the baseline, 4 weeks, and surgery time points. All tissues are in analysis. This is a particularly important trial in that these patients are previously untreated and as primary tumors represent a much more homogeneous source of tumor material in which to compare pre- to post-treatment changes.

Task 1: Trial II: Randomized Preoperative trial in AR+/ER+ BC (Elias, LoRusso, Traina, advocates, Richer)

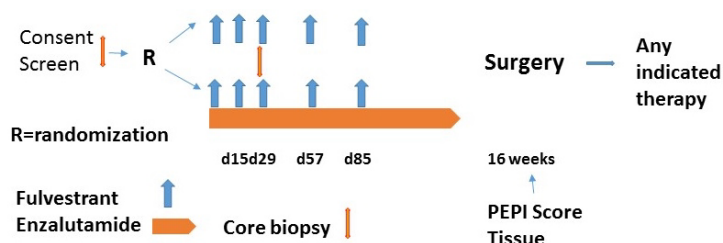
- Written protocol completed Month 21
- Submitted to Scientific Review Committee Month 21
- Submitted to IRBs (all institutions) Month 22
- DoD Human Research Protection Office (HRPO) Month 24

The Clinical Aim 4 trial was originally a randomized phase II trial of exemestane +/- enzalutamide given preoperatively for women with T2 or larger AR+/ER+ BC. This LOI was submitted to Astellas/Medivation in August 2015. Before it was evaluated, Astellas shut down their IIT program pending an internal assessment of their development plans for enzalutamide in breast cancer. This LOI was ultimately evaluated and rejected in 12/2015. An LOI for the same trial using fulvestrant +/- enzalutamide was submitted in 1/2016 and accepted by Astellas in 2/2016. The full protocol was written and submitted to Astellas and to the UCCC Scientific Review Committee in May 2016. Because >90% of ER+ BC is also AR+, AR IHC is not being used to select patients.

- Found to be IND exempt by FDA in 8/2016
- COMIRB approved 9/2016 – Protocol 16-1042
- HRPO approved 4/2017
- Astellas approved 10/2016
- Astellas Drug Supply contract and IP agreement approved 8/2017
- Trial activated at University of Colorado 8/30/17
- Patient #1 accrued 10/2/17
- Trial open to accrual at MSKCC 7/2/18
- Trial open to accrual at University of Tennessee 9/17/18
- 69 patients consented. 62 enrolled (3 screen failures and 4 withdrawals prior to enrollment), and 61 randomized and treated (1 patient withdrew prior to treatment). Stage I accrual was completed 3/2/20, with 22 evaluable subjects enrolled on the experimental (combination) arm. More than 4 patients on the combination arm have achieved a PEPI score = 0, therefore an additional 16 patients were enrolled in stage 2. Stage 2 accrual was completed on 10/14/2021. 5 year follow-up for relapse and survival is continuing as per approved protocol.

Preoperative Fulvestrant +/- Enzalutamide

Eligibility: $\geq$ T2; ER+/Her2-



If pre- or perimenopausal, goserelin 3.6 mg sq every 4 weeks (or equivalent)

Samples to be collected:

- Tumor tissue: at baseline, at 4 weeks, at time of surgery (16 weeks)
- Plasma: at baseline, at 4 weeks, at time of surgery (16 weeks)

- SAEs that were possibly related: G4 MI (1)

- SAEs felt to be unrelated: G3 gallbladder obstruction (1); G3 hematoma from breast surgery

Primary endpoint: To evaluate whether the addition of enzalutamide to fulvestrant treatment for ~4 months in women with $\geq$ T2 ER+/Her2- BC will achieve a PEPI score of 0 at time of surgery in 32%. PEPI (preoperative endocrine prognostic index) is a model that combines ER, pathologic

tumor site, nodal status, and Ki67 score at time of surgery to predict subsequent risk of recurrence. PEPI = 0 means ypT < 1cm; Ki67 < 10%; N0; ER+. PEPI is used to guide postoperative therapy in a number of neoadjuvant breast cancer trials ([NCT01723774](#), [NCT02236572](#), [NCT01923168](#), [NCT01953588](#)). Fulvestrant has been shown to be more active against ER+ MBC than AIs in the randomized phase II FIRST trial. Phase III confirmation of fulvestrant vs. AI in MBC in the FALCON trial ([NCT01602380](#)). The ALTERNATE trial, accruing in NCTN, randomizes women with cT2-4 N0-3 M0 ER+/Her- breast cancer to anastrozole, fulvestrant, or the combination for 6 months prior to surgery ([NCT01953588](#)).

Statistical design: From the literature, preoperative aromatase inhibitor (AI) achieved PEPI score of 0 in 16%. We expect to achieve PEPI score of 0 in 32% for fulvestrant plus enzalutamide. Fulvestrant alone would likely be intermediate. We are using a Simon 2-stage design for the combination (experimental) arm: if ≤ 3 PEPI = 0 in first 22 evaluable patients, then will terminate entire trial. If ≥ 4 achieve PEPI = 0, then will increase arm size to 34. The probability of early termination is 0.52. We will have an 80% power with a type I error rate of 0.08. The trial has a concurrent control arm of fulvestrant alone with 27 patients. If the true PEPI = 0 is $>16\%$, the observable rate has a 90% likelihood of being $> 6\%$. We anticipate $\sim 10\%$ inadequate tissue specimens. A total of 49-61 patients will be randomized and treated.

Grant Hypotheses (from serial tissue biopsies):

- Certain pretreatment molecular characteristics (such as AR:ER ratio in ER+ tumors, Her2 status, PI3K pathway mutations, or others) will be associated with lesser response and poorer PEPI score (Clinical Aim 1).
- Decrease in Ki67 after ~ 4 weeks of treatment to below 10% will be associated with response to therapy and will correlate with improved PEPI scores (Clinical Aim 2).
- High AR expression will be associated with resistance to anti-estrogen therapy. Its blockade may enhance response (Clinical Aim 3).

Eligibility: At least 18 years of age, ER+/Her2- BC ($>90\%$ will be AR+), stage $\geq cT2$, planned to get local surgery, PS 0-2, safe to biopsy, no prior treatment. Women must be postmenopausal, or if pre- or peri-menopausal, will require concurrent ovarian suppression.

Clinical Results: Overall 69 patients were consented, of whom 59 were evaluable (33 on combination arm and 26 on the fulvestrant only arm). Upon completion of the Simon stage I portion of the experimental arm, 4/22 patients had achieved PEPI = 0, whereupon the second stage completed accrual. Of the 33 patients treated on the FE arm, median age was 63. At baseline, 21 had T3/T4 tumors, 91% were ER+/PR+, median AR expression was 80%, median Ki67 was 15%. Of the 26 patients treated on the F arm, median age was 61, 19% had T3/T4 tumors, 96% were ER+/PR+, median AR expression was 85%, median Ki67 was 10%. Toxicity was as expected with endocrine therapy. Surgery was completed in 95% and PEPI = 0 achieved in 10/59 (17%). PEPI=0 was achieved more frequently on the FE arm (8/33, 24%) than the F arm (2/26, 8%) ($p = 0.16$). Table 1.

Table 1: Summary Statistics

	Combination Arm (N=33)	Fulvestrant Arm (N=26)	Total (N=59)
Age at Consent (Years)			
N	33	26	59
Median (Range)	63 (41, 78)	61 (32, 83)	63 (32, 83)
ECOG PS			
N	33	26	59
Median (Range)	0 (0, 1)	0 (0, 2)	0 (0, 2)
T Stage			
N	33	26	59
T2	26 (78.8%)	21 (80.8%)	47 (79.7%)
T3	6 (18.2%)	5 (19.2%)	11 (18.6%)
T4	1 (3.0%)	0 (0.0%)	1 (1.7%)
AR%*			
N	27	22	49
Median (Range)	80 (10, 100)	85 (10, 100)	80 (10, 100)
ER and PR*			
N	33	26	59
ER+ & PR-	3 (9.1%)	1 (3.8%)	4 (6.8%)
ER+ & PR+	30 (90.9%)	25 (96.2%)	55 (93.2%)
Baseline Lab Ki67 (%)			
N	29	21	50
Median (Range)	15 (1, 80)	10 (1, 60)	12 (1, 80)
Surgery**			
N	33	26	59
No	2 (6.1%)	1 (3.8%)	3 (5.1%)
Yes	31 (93.9%)	25 (96.2%)	56 (94.9%)
PEPI Score (p=0.16)***			
N	33	26	59
>0	25 (75.8%)	24 (92.3%)	49 (83.1%)
0	8 (24.2%)	2 (7.7%)	10 (16.9%)
Follow-Up Time (Months)****			
N	33	26	59
Median (Range)	3.7 (0.9, 4.7)	3.6 (2.5, 5.8)	3.7 (0.9, 5.8)

*Lab data were used for AR% and clinical data were used for ER and PR. All patients were AR positive.

**"Yes" indicates patients who had surgery during the study and "No" indicates patients who did not have surgery during the study. The 3 (100%) patients who did not have surgery also did not complete at least 4 months of therapy.

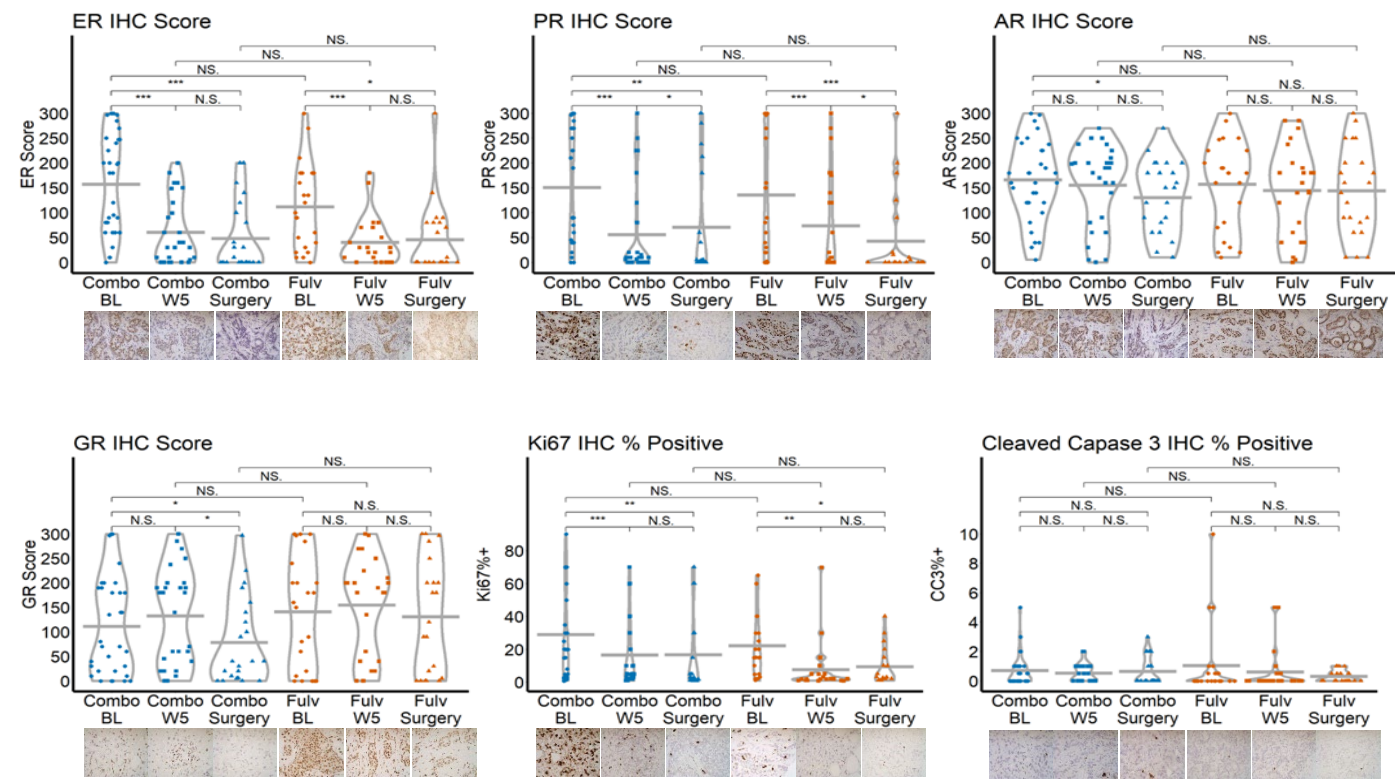
***Fisher's exact test was used to calculate the p-value comparing PEPI score across arms

****Follow-Up Time is defined as the time in months from the start of Fulvestrant until the treatment was completed per the protocol or censoring. 1 (1.7%) patient was censored due to an Adverse Event.

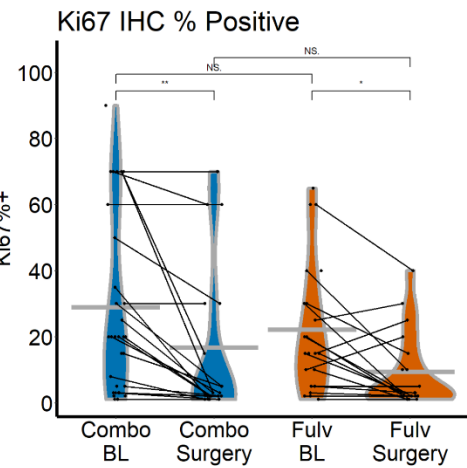
IHC comparison of Baseline and Week 5 biopsies showed decreased ER, PR, and Ki67 levels that remained decreased at time of surgery. AR and GR were significantly decreased only by the time of surgery and only in the combination Fulvestrant plus Enzalutamide arm.

Figure 1. Immunohistochemistry for the estrogen, progesterone, androgen and glucocorticoid receptors (ER, PR, AR, GR), Ki67 and Cleaved Caspase 3 in serial biopsies by treatment arm and time (baseline, week 5 and at time of surgery). ER, PR and Ki67 decreased in both the combination and fulvestrant only arm. AR significantly decreased by time of surgery only in the combination arm, as did GR. Points represent individual observations and differences in the mean in

each group are indicated by a horizontal line. "Combo," "Fulv," "BL," and "W5," indicate combination arm, fulvestrant arm, baseline timepoint, and week 5 timepoint. T-tests compared the combination and fulvestrant arms for each outcome and timepoint. Paired t-tests were used to evaluate within-group changes over time. "N.S.," "**," "***," and "****" indicate non-significant, ($p>0.05$), $p<0.05$, $p<0.01$, and $p<0.001$. P-values were not adjusted for multiple testing.gExample of one patient's paired samples over time are shown for each protein. Magnification =400x



Ki67 significantly decreased in both the combination Fulvestrant plus Enzalutamide arm of the study and the Fulvestrant only arm. Importantly, no patient's tumor Ki67 increased in the combination group, while some tumors did increase in the fulvestrant only group.



	Combination Arm (N=21)	Fulvestrant Arm (N=17)	Total (N=38)	p value
IHC Ki67 % Pos Group*				
High BL, High Surgery	7 (33.3%)	6 (35.3%)	13 (34.2%)	1.0
High BL, Low Surgery	8 (38.1%)	6 (35.3%)	14 (36.8%)	
Low BL, Low Surgery	6 (28.6%)	5 (29.4%)	11 (28.9%)	

Figure 2. Ki67 significantly decreased in both the combination Fulvestrant plus Enzalutamide arm of the study and the Fulvestrant only arm. Left: Violin plot of Ki67% by time and treatment arm. Lines connect same patient over time at baseline "BL," and time of surgery. T-tests compare combo and fulvestrant arms for each outcome and "N.S.," "**," "***" indicate non-significant $p<0.05$ and $p<0.01$. Paired t-tests were used for within-group changes over time. Right: Patients were categorized into groups based on IHC Ki67 percent positive at the BL and surgery timepoints. Patients missing Ki67 at the surgery timepoint were eliminated. "Low" indicates Ki67 <10% positive and "High" indicates

Ki67 $\geq 10\%$. Importantly, no patient's tumor Ki67 increased in the combination group, while some tumors did increase in the fulvestrant only group. However, overall there was not a significant difference between the two arms.

RPPA revealed interesting differences in phosphoproteins across groups. Figure 3 depicts those that were differentially Altered by Treatment in Tumors that Responded (Ki67 reduced to less than 10%) versus those where Ki67 remained elevated. For some of the dramatic differences we can examine whether the differences at time of surgery (4 months) will be stranger than they were at these week 5 timepoints.

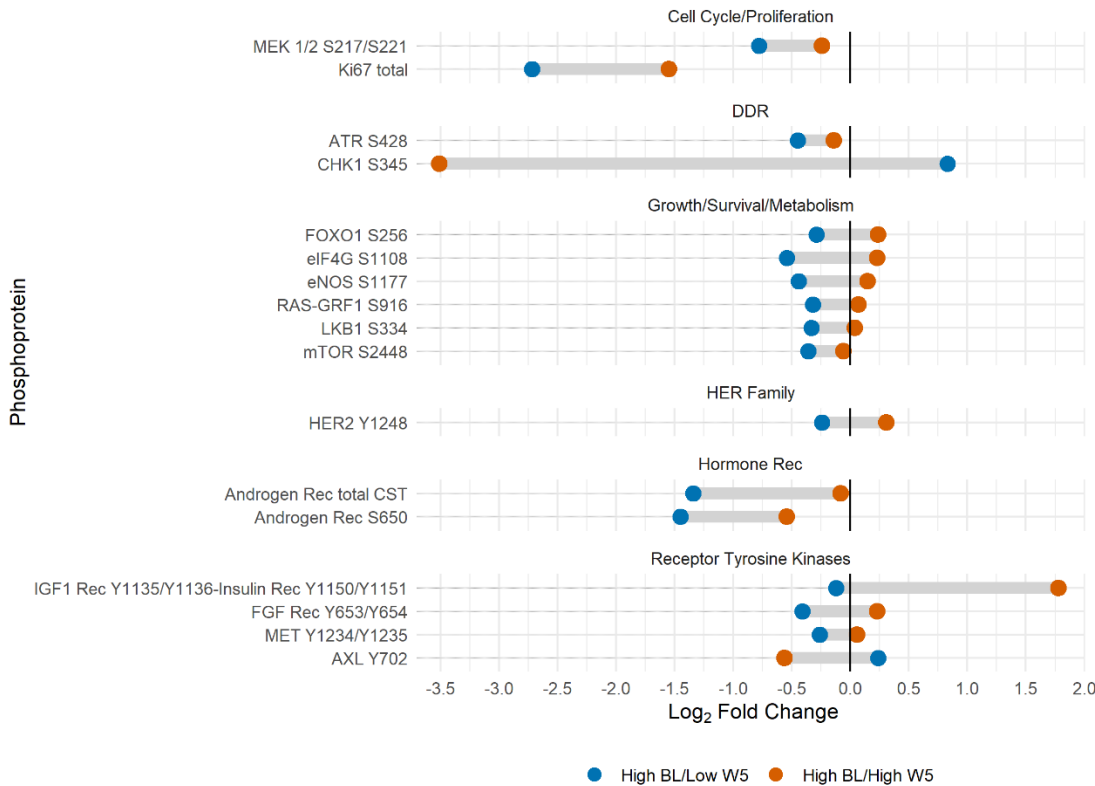


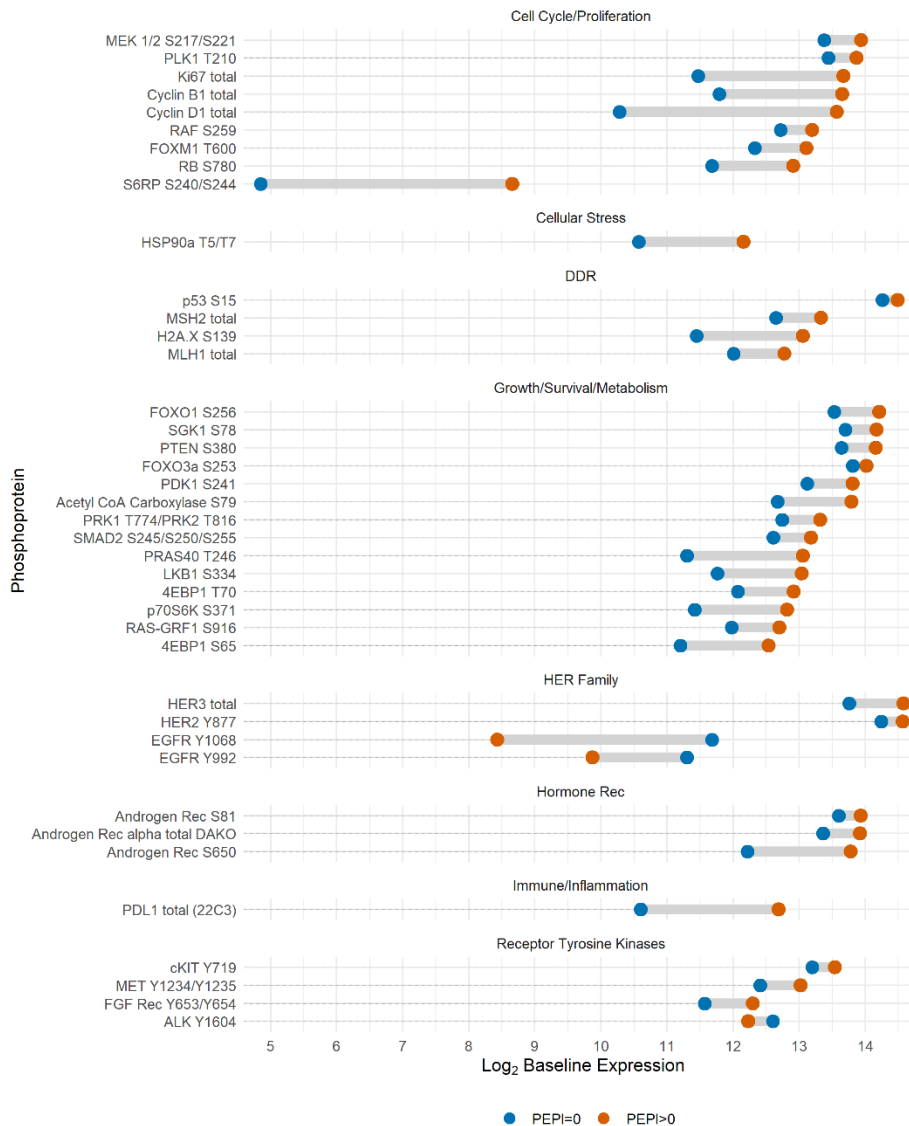
Figure 3. Tumors with a significant decrease in Ki67 had a significant reduction in AR total and phospho S650AR with treatment, while those where Ki67 remained high had decreased CHK1 and elevated phosphoIGF1 receptor. Left: Dumbbell plot of the mean log₂ change in expression among patients with High BL/High W5 (blue) and High BL/Low W5 (orange) ($p < 0.1$) phosphoprotein. Positive changes indicate increases in expression and negative changes indicate decreases. Right: Phosphoproteins with $p < 0.2$ are presented in log₂ units with the mean change and SD among patients with

High BL/Low W5 and High BL/High W5 in the 2nd and 3rd columns. Positive indicates average within-group increases in expression over time and negative means indicate average within-group decreases in expression over time. "Mean Diff" is the mean change among patients with High BL/High W5 (column 3) subtracted from the mean change among patients with High BL/Low W5 (column 2).

We then examined **Proteins Differentially Expressed at Baseline in Tumors that Achieved PEPI=0 vs PEPI>0**. Of course proliferative signals were higher in the tumors that did not achieve PEPI=0. The mTOR pathway and other growth signals were higher pretreatment in tumors with poor histologic response, an observation also observed in our trial of FE in pts with ER+/HER2- metastatic disease (SABCS 2021). Likewise, baseline PD-L1 and AR were higher in tumors that did not achieve PEPI=0, particularly AR phosphorylated on Serine 650. AR signaling is known to support immunosuppressive cells in the microenvironment, and we observed a marked decrease in MDSCs with treatment only on the FE arm (AACR 2023 poster and manuscript in preparation) shown later in this report. Interestingly, EGFR pathway activation pEGFR (Y1604 and Y992), were the only phospho proteins higher in pretreatment tumor of patients achieving PEPI=0 (Figure 4).

Figure 4. PhosphoEGFR (Y1604 and Y992) higher in tumors that had a good response (PEPI=0), whereas cell proliferation, metabolism and mTOR pathway activation were higher in PEPI>0 tumors as were PD-L1 and AR. To identify phosphoproteins DE at baseline (BL) between tumors that responded to treatment (PEPI=0) versus those that did not (PEPI>0) Empirical Bayes moderated t-tests were used to calculate unadjusted p-values. Left: Dumbbell plot of mean

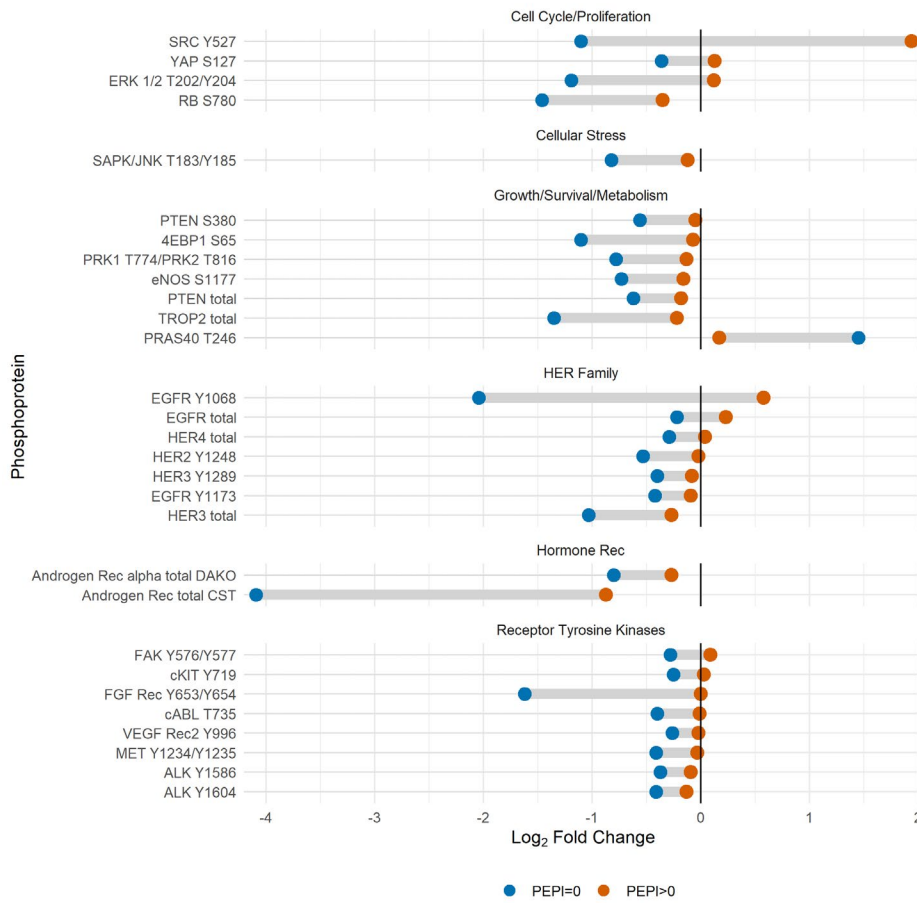
$\log_2$ baseline expression in PEPI=0 (blue) versus PEPI>0 (orange) tumors for each significantly different ($p<0.1$) phosphoprotein. Right: Proteins $p<0.2$ are presented in $\log_2$ units with the mean and standard deviation (SD) among PEPI=0 and PEPI>0 tumors in the 2nd and 3rd columns, respectively. “Mean Diff” is the mean among PEPI>0 (column 3) subtracted from the mean with PEPI=0 (column 2).



When comparing phosphoproteins **Differentially Altered by Treatment (Changed from Baseline to Week 5)**, the total **AR** by RPPA was the most differentially decreased protein in tumors that responded the best (achieved PEPI=0 status). **EGFR Y1068** and **FGFR Y653/Y577** also decreased significantly after 5 weeks of treatment. Interestingly, **SRC Y527** decreased in tumors achieving PEPI=0, but actually increased with treatment in the non-responsive tumors. In the future we can purchase phospho specific antibodies to these proteins that changed the most in responsive versus non-responsive tumors after 5 weeks of treatment and determine by IHC how much they changed by the time of surgery. We know that by IHC, the AR decrease at the protein level was not significant until time of surgery and only in the combo arm (Figure 1).

Figure 5. Among tumors that achieved PEPI=0, there was a decrease in AR, EGFR Y1068, FGFR Y653/Y577 and SRC Y527 after 5 weeks of treatment. To assess whether the average change in expression (Week5-Baseline) among tumors with PEPI=0 was significantly different ($p<0.1$) from PEPI>0 tumors, the group-specific mean change was calculated for

each phosphoprotein and empirical Bayes moderated t-tests used to calculate unadjusted p-values. Left: Dumbbell plot of the mean $\log_2$ change in expression among patients with PEPI=0 (blue) and PEPI>0 (orange) for each significant ($p<0.1$) phosphoprotein. Positive changes indicate increased expression and negatives indicate decreased expression. Right: Phosphoproteins with $p<0.2$ are presented in $\log_2$ units with the mean change and standard deviation (SD) among patients with PEPI=0 and PEPI>0 in the 2nd and 3rd columns, respectively. Positive means indicate average within-group increases in expression over time and negatives indicate average within-group decreases over time. “Mean Diff” = mean change among PEPI>0 (column 3) subtracted from the mean change PEPI=0 (column 2).



Conclusions from the RPPA analysis:

- PEPI=0 was achieved more frequently on the combination arm (8/10) than Fulvestrant only arm (2/10), although this difference ($p=0.16$) did not meet the pre-specified statistical significance.
- No new safety concerns were observed, most grade 1. Mild reversible cognitive impairment and occasional tremor were associated with Enzalutamide (also observed in prior Enzalutamide trials).
- IHC comparison of Baseline and Week 5 biopsies showed decreased ER, PR, and Ki67 levels that remained decreased at time of surgery. AR and GR were significantly decreased only by the time of surgery and only in the combination Fulvestrant plus Enzalutamide arm.
- RPPA showed a significant reduction in AR total and pS650AR with treatment in tumors where Ki67 decreased to less than 10%, while tumors in which Ki67 remained high had low CHK1 S345 and elevated phosphoIGF1 receptor.
- RPPA identified AR, EGFR Y1068, FGFR Y653/Y577 and SRC Y527, and as proteins most differentially decreased by treatment in tumors with PEPI=0 as compared to PEPI>0 tumors.

AR signaling is known to support immunosuppressive cells in the microenvironment, and we observed a marked decrease in myeloid derived suppressor cells (MDSCs) with treatment only in the combination Fulv plus Enzalutamide arm (manuscript in preparation). We asked what changes are observed in the immune microenvironment with treatment? We used Akoya Polaris Multiplex Immune Panel P9HuP58 HIMSR Core Panel with the following markers:

CD14+HLADR+CD68- = Monocytes
 CD14+HLADR-CD68- = Myeloid-derived suppressor cells
 CD68+CD163- = M1 macrophages
 CD68+CD80+CD163- = M1 subset co-stim T cell
 CD68+CD163+ = M2 macrophage
 CD209+CD80+HLADR+ = Dendritic cells
 CD209+CD80+ = Dendritic cells
 CD209+HLADR+ = Dendritic cells

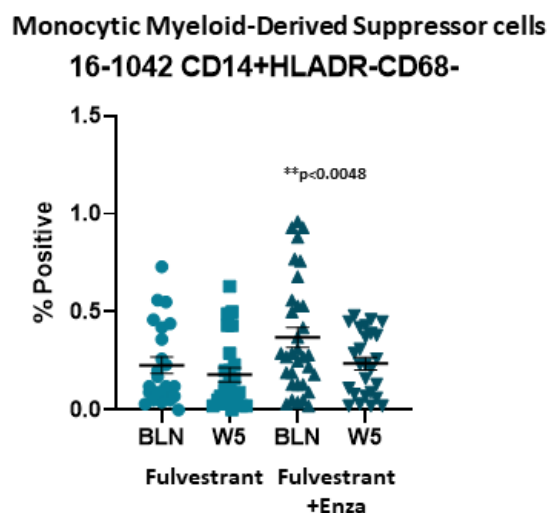
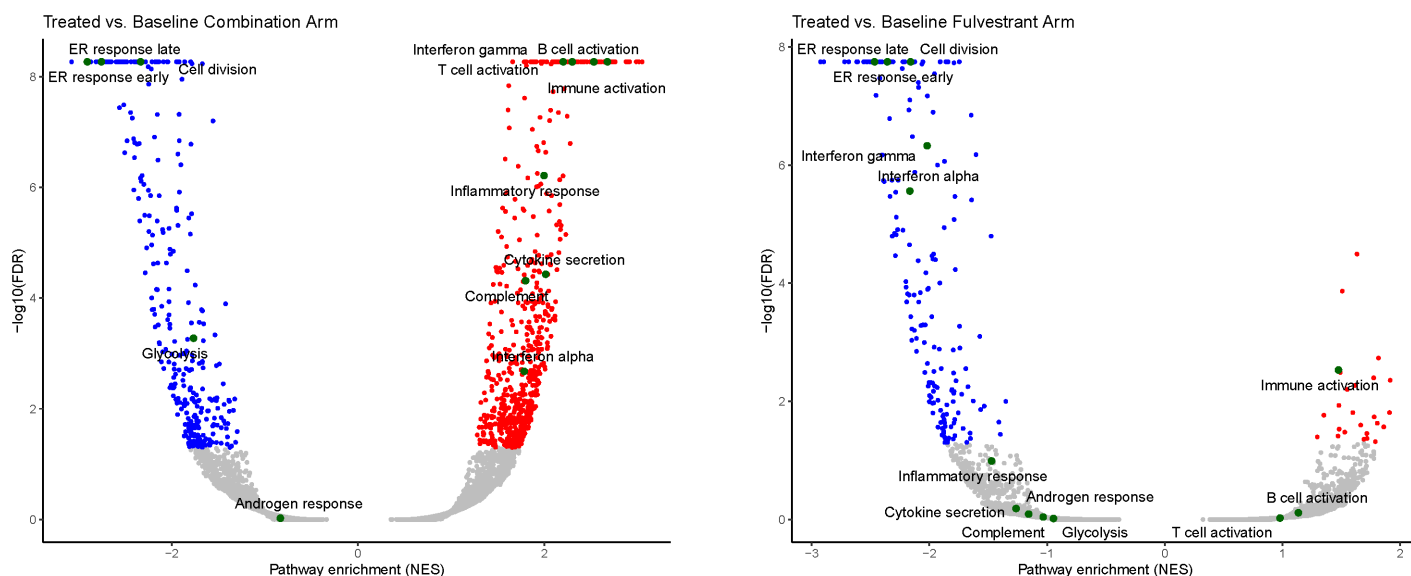


Figure 6. Akoya Polaris Multiplex Immune Panel P9HuP58 HIMSR Core Panel demonstrated that MDSCs decreased with treatment only in the combination Fulv plus Enzalutamide arm. The following markers were used: CD14+HLADR+CD68- = Monocytes
 CD14+HLADR-CD68- = Myeloid-derived suppressor cells
 CD68+CD163- = M1 macrophages
 CD68+CD80+CD163- = M1 subset co-stim T cell
 CD68+CD163+ = M2 macrophage
 CD209+CD80+HLADR+ = Dendritic cells
 CD209+CD80+ = Dendritic cells
 CD209+HLADR+ = Dendritic cells

We performed Biospyder gene expression analysis and found interesting differences between the Baseline versus Week 5 combination Fulvestrant plus enzalutamide arm compared to the Fulvestrant only arm (Figure 7). A difference in immune activation in the combination arm included a Interferon gamma and Interferon alpha and inflammatory pathway response that was not observed in the fulvestrant only arm. This could possibly explain a decrease in what we believe are MDSCs or be the reason for more PEPI=0 in the combination arm versus the fulvestrant only arm (8 versus 2).

Figure 7. Gene set enrichment analysis of Biospyder gene expression data demonstrated interesting differences between the pretreatment biopsy (Baseline) versus Week 5 in the combination Fulvestrant plus enzalutamide arm compared to the Fulvestrant only arm. The analysis was performed with Hallmark and Gene Ontology Biological Processes gene set collections using the fgsea R package on the fold-change for the indicated comparisons. Normalized enrichment scores (positive (red) indicates upregulated in treatment group) and FDR are plotted for each treatment arm with select gene sets labeled.



Clinical Aim 5: To determine the maximum tolerated dose and toxicity of enza when combined with the most promising combinations as defined in the preclinical modeling experiments during Years 1-2.

Task 1: Trial III: Phase II trial in ER+ metastatic BC: Enzalutamide plus everolimus

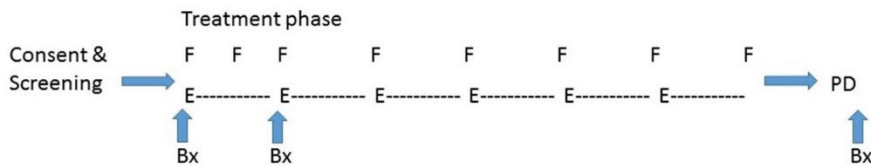
- Written protocol completed Month 21
- Submitted to Scientific Review Committee Month 21
- Submitted to IRBs (all institutions) Month 22
- DoD Human Research Protection Office (HRPO) Month 24

Support for the original trial of enzalutamide plus everolimus for AR+ TNBC was not granted by Medivation and Astellas. An alternative trial, as presented at the Milestone Meeting in May 2015, was a Phase I/II trial in ER+ BC: Enzalutamide plus exemestane or fulvestrant with the addition of either everolimus or palbociclib. This LOI was submitted to Medivation and Astellas and was rejected for support or drug supply. Ultimately, once the phase I of fulvestrant plus enzalutamide was completed and reported, the following phase II trial was approved, then submitted for an FDA IND application in 8/2016. It was declared IND exempt by CTEP.

- **IRB (COMIRB 16-1001) approved 10/2016**
- HRPO approved 4/2017
- Submitted to regulatory committee at University of Tennessee 11/2016
- Astellas approved 10/2016
- Astellas Drug Supply contract and IP agreement approved 6/2017
- Trial activated at University of Colorado 6/29/17
- Trial activated at University of Tennessee 9/17/18
- Monthly teleconferences to review patients, safety, and research progress. Also have a trial monitor (Dr. Lam).
- Accrual completed November 2019 after reaching goal of 20 evaluable subjects
- SAEs felt to be possibly related: None

- SAEs felt to be unrelated: G2 pleural effusion (2), G3 seizure (1), G3 fracture (1); G3 pain (2); G2 gallbladder obstruction (1); G3 increased AST (1); G4 hypercalcemia with hypokalemia, anemia and fatigue (1); G3 overdose; G3 dehydration and UTI (1); G3 biliary obstruction (1); G2 basal cell carcinoma right lower eyelid (1); G3 vomiting with G4 hypotension

Phase II trial of fulvestrant plus enzalutamide in ER+/Her2- advanced breast cancer



F Fulvestrant 500 mg IM (1st month with SOC loading schedule)

E Enzalutamide 160 mg PO daily

Fulvestrant may start concurrently with E or up to 3 months before E

Bx Tumor Biopsy (3rd one optional)

If pre- or perimenopausal, goserelin 3.6 mg sq every 4 weeks (or equivalent)

Samples to be collected:

- Tumor tissue: at baseline, at 4 weeks, at time of surgery (16 weeks)
- Plasma: at baseline, at 4 weeks, at time of surgery (16 weeks)

Primary Endpoints: To determine the clinical benefit rate (CBR) of adding enzalutamide to fulvestrant treatment in women with ER+/Her2- advanced BC. To evaluate the safety and tolerability of fulvestrant plus enzalutamide.

Statistical Design: Open label single arm phase II trial with 24 patients. Undesirable clinical benefit rate (CBR at 24 weeks) would be $\leq 10\%$. Desirable CBR would be $\geq 30\%$. This would provide 89% power with a one-sided α of 0.085. If $\geq 5/24$ patients have CBR of ≥ 24 weeks,

then combination warrants further evaluation. We anticipate 10% inadequate tissue specimens.

Grant Hypotheses (from serial tissue biopsies):

- Decrease in Ki67 after ~4 weeks of treatment to below 10% will be associated with response to therapy and will correlate with CBR (Clinical Aim 2).
- Pretreatment molecular characteristics (such as AR:ER ratio in ER+ tumors, Her2 status, PI3K pathway mutations, or others) will be associated with CBR (Clinical Aim 1).
- High AR expression will be associated with resistance to anti-estrogen therapy. Its blockade may enhance response (Clinical Aim 3).
- Tissue at time of disease progression will be enriched for genes/proteins/mutations representing resistance mechanisms.

Eligibility: At least 18 years of age, ER+/Her2- BC (>90% will be AR+), metastatic, tumor tissue available and safe for serial biopsy, candidate for fulvestrant therapy, PS 0-2, safe to biopsy, no CNS disease. Women must be postmenopausal, or if pre- or peri-menopausal, will require concurrent ovarian suppression.

Conclusions:

The manuscript reporting the results of the trial for highly pretreated metastatic breast cancer was accepted to NPJ Breast Cancer (PMID: 37210417). We also published two papers before the trial was completed by examining the pretreatment specimens to look for differences in immune cell status in the different specimens of metastatic breast cancer with different mutations from patients with highly resistant metastatic breast cancer that were highly pretreated prior to going on our trial. The first paper demonstrated that steroid hormone receptor and infiltrating immune cell status revealed therapeutic vulnerabilities of ESR-1-mutant breast cancer (Cancer Res 2021; PMID: 33184106) and the second, that that another showing mutual exclusivity of ESR1 and TP53 mutations in endocrine resistant metastatic breast cancer (NPJ Breast Cancer. 2022 PMID: 35538119)

Summary of results from the trial from the now published manuscript (PMID: 37210417: Of the 32 eligible participants, 28 were evaluable for response. The median age was 61 [46-87] years and the median ECOG PS was 1 [0-1]. Patients were heavily pretreated with a median of 3 [1,9] prior hormonal agents and 4 [0,8] prior non-hormonal therapies. Twelve (37.5%) patients had prior fulvestrant and 29 (90.6%) had visceral disease. Treatment emergent adverse events were consistent with what would be expected with hormonal therapy. At week 24, 7 (25.0%) (95% CI: 10.7 to 44.9) participants had stable disease and no participants had partial response. The median time to progression was 8 weeks (95% CI: 8 to 20).

Significant ($p < 0.2$) univariate relationships were observed between continuous PFS and the following variables: ER and AR percent positivity by IHC, and PIK3CA and/or PTEN mutations. Specifically, the average hazard for progression was 4.32 (95% CI: 1.46 to 12.83) times higher for participants with ER < 10 percent positive compared to participants with ER $\geq$ 10 percent positive ($p = 0.008$). This association remained significant when ER was considered continuous ($p = 0.024$). Additionally, the average hazard of progression was 2.28 (95% CI: 0.93 to 5.62) times higher for participants with AR < 10 percent positive compared to participants with AR $\geq$ 10 percent positive ($p = 0.073$). The hazard of disease progression for participants with AR and/or ER < 10% positive was 2.35 (95% CI: 0.99 to 5.58; $p = 0.052$) times the hazard for participants with AR and/or ER $\geq$ 10% positive.

When comparing phosphorylated proteins in the in the “Short PFS” defined in all RPPA analyses as PFS $\leq$ 60 days ($n = 20$) to those who experienced “Long PFS” defined as PFS > 24 weeks (168 days) ($n = 7$), the mTOR pathway had higher baseline expression levels the tumors of those with Short PFS. According to robust moderated t-tests, baseline mTOR S2448 ($p = 0.008$), eNOS/NOSIII S116 ($p = 0.029$), S6RP S240/S244 ($p = 0.031$), eIF4G S118 ($p = 0.037$), and p70S6K T389 ($p = 0.043$) were significantly higher in the Short PFS group compared to the Long PFS group. Similar results were obtained when Short PFS was defined as < 24 weeks ($n = 25$). This suggests mTOR activation in the pre-treatment tumor biopsies from patients with a short time to progression.

Future trials could be done in second line endocrine therapy (progressing after AI and cdk4/6 inhibition) and in PI3K wild type tumors to evaluate fulvestrant plus everolimus with or without an AR signaling blocker.

From the neoadjuvant trial, conclusions are that the combination of Fulvestrant plus Enzalutamide (F plus E) had manageable side effects. PEPI=0 was achieved more frequently on the combination arm F plus E (8/33) than the F only arm (2/26), although this difference ($p = 0.16$) did not meet the pre-specified statistical significance. It is notable that we did not observe evidence for tumor stimulation with AR blockade. HC comparison of Baseline and Week 5 biopsies showed decreased ER, PR, and Ki67 levels that remained decreased at time of surgery. AR and GR were significantly decreased only by the time of surgery and only in the combination Fulvestrant plus Enzalutamide arm. RPPA showed a significant reduction in AR total and pS650AR with treatment in tumors where Ki67 decreased to less than 10%, while tumors in which Ki67 remained high had low CHK1 S345 and elevated phosphoIGF1 receptor. RPPA identified AR, EGFR Y1068, FGFR Y653/Y577 and SRC Y527, and as proteins most differentially decreased by treatment in tumors with PEPI=0 as compared to PEPI>0 tumors. AR signaling is known to support immunosuppressive cells in the microenvironment, and we observed a marked decrease in myeloid derived suppressor cells (MDSCs) with treatment only in the combination Fulv plus Enzalutamide arm, indicating that perhaps the anti-androgen reduced these number of these cells. Interestingly, gene expression analysis did show an increase in immune activation that was observed only in the combination arm.

Publications, Abstracts, and Presentations:

Publications:

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2. Barton VN, D'Amato NC, Gordon MA, Lind HT, Spoelstra NS, Babbs B, Heinz RE, Elias AD, Jedlicka P, Jacobsen BM, Richer JK. Multiple molecular subtypes of triple negative breast cancer depend on androgen receptor for proliferation and invasion. *Molecular Cancer Therapeutics* 2015; 14: 769-778. PMID: 25713333
3. Barton VN, Gordon MA, Christenson JL, D'Amato NC, Elias A, Richer JK. Androgen receptor biology in triple negative breast cancer: a case for AR+ and quadruple negative disease subtypes. *Horm Cancer* 2015 Jul 23, epub ahead of print. PMID: 26201402.
4. D'Amato NC, Gordon MA, Babbs B, Spoelstra NS, Carson Butterfield KT, Torkko KC, Phan VT, Barton VN, Rogers TJ, Sartorius CA, Elias A, Gertz J, Jacobsen BM, Richer JK. Cooperative Dynamics of AR and ER Activity in Breast Cancer. *Mol Cancer Res.* 2016; 14: 1054-1067. PMID: 27565181 (Figure on cover).
5. Barton VN, Christenson JL, Gordon MA, Greene LI, Rogers TJ, Butterfield K, Babbs B, Spoelstra NS, D'Amato NC, Elias A, **Richer JK**. Androgen receptor supports an anchorage-independent, cancer stem cell-like population in triple-negative breast cancer. *Cancer Res* 2017; 77(13):3455-3466. PMID: 28512248
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Abstracts:

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Oral Presentations by Richer related to this research:

- | | |
|------------|--|
| Oct 2013 | AACR Advances in Breast Cancer Research Conference Invited Symposia Lecture "Targeting the Androgen Receptor in Breast Cancer", Targeted Therapies San Diego, CA. |
| Jan 2014 | Keystone Symposia on Nuclear Receptors: Biological Networks, Genome Dynamics and Disease. Invited short talk: Role of Androgen Receptors in Estrogen Receptor Negative Breast Cancer. Taos, NM. |
| March 2014 | Society for Gynecologic Investigation. Invited Symposia Lecture "Functional Significance of miRNAs Contributing to Reproductive Cancers" Florence, Italy, |
| Sept 2015 | US Oncology Mckesson Annual Scientific Forum Invited Lecture "Role of Androgen Receptors in Breast Cancer" Dallas, TX |
| Jan 2016 | Keystone Symposium Nuclear Receptors Scientific organizer and speaker "Subtype-Specific AR Action in Breast Cancer" Snowbird, Utah. |
| April 2016 | Endocrine Society Annual Meeting 2016 Invited Symposia Lecture "Role of Androgen Receptors in Breast Cancers Resistant to Estrogen Receptor-Directed Endocrine Therapies" Boston, MA |

May 2016	Gordon Research Conference on Mammary Gland Biology and Cancer Short talk “Targeting the Androgen Receptor in Triple Negative Breast Cancer, Lucca, Italy
June 2017	FASEB SRC Meeting on Rapid Signaling & Genomic Hormone Action in Health & Disease. Invited oral presentation on “Androgen Receptor and mTOR Cross Talk in Breast Cancer”
Oct 2017	Breast Cancer Research Foundation Think Tank for Androgen Receptor in Breast Cancer
Oct 2017	Breast Cancer Research Foundation Think Tank for Androgen Receptor in Breast Cancer
Dec 2017	San Antonio Breast Cancer Symposium. Invited Educational Session presentation “Androgen Receptors in Breast Cancer” Symposium on Androgen, Progesterone and Glucocorticoid Receptors: Reprogramming of Steroid Receptors during Breast Tumor Progression
April 2018	AACR Invited “Meet the Expert” session “Update on Potential for Targeting Androgen Receptors in Breast Cancer.”
March 2018	Endocrine Society invited Symposium presentation: Steroid Receptors and Inflammation titled “Triple-Negative Breast Cancer Hijacks a Developmental Program of Immune Suppression”
Aug 2019	Gordon Research Conference Hormones and Cancer , Sunday River, Maine, “Hormone Deprivation Influences Breast Cancer Immune Suppression”
Sept 2020*	7th Annual Metastatic Breast Cancer Conference , Huntsman Cancer Institute, Salt Lake City, UT. “Targetable Pathways in metastatic ER+ BC resistant to aromatase inhibitor therapy”
*Oct 2020	National Institute of Environmental Health Sciences NIEHS Reproductive & Developmental Biology Laboratory (RDBL) “Steroid Hormone Milieu and female cancers: context is critical.” *remote due to Covid19
*Oct 2021	The University of North Carolina Chapel Hill Pathology Laboratory Medicine Molecular and Cellular Pathology “Steroid Hormone Milieu and female cancers: context is critical
June 2023	Endocrine Society Annual Mtg <i>Androgen Control of Mitochondrial Function in breast cancer</i>
April 2023	Georgetown University Medical Center Dean’s Seminar Series Identifying and exploiting vulnerabilities in breast cancer resistant to traditional endocrine therapy.

Inventions, Patents and Licenses:

US Patent Title: FKBP52-SPECIFIC ANDROGEN RECEPTOR INHIBITOR, MJC13, FOR USE IN TREATING BREAST CANCER

- Provisional Patent Application: 62/350,383
- Non-Provisional Patent Application: 15/623,775
- Patent Number: 10,434,075

Reportable Outcomes:

Metastatic trial 16-1001 manuscript published in npj Breast Cancer (PMID: 372104170). Preoperative trial 16-1042 poster at AACR 2023, and manuscript under preparation. Please see list of 13 published manuscripts

Other Achievements:

Website(s) or other Internet site(s)

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Expert Opinion piece in Oncology PracticeUpdate <http://www.practiceupdate.com/journalscan/9370> or http://prac.co/j/5960d32c-988b-423e-ba24-14ca5c8cc39a?elsca1=soc_share-this acknowledgement of federal support –no

Highlight of Cochrane DR et al Breast Cancer Research 2014 in Feb issue of 2014 NATURE REVIEWS CLINICAL ONCOLOGY. acknowledgement of federal support –yes

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ARTICLE OPEN



Phase II trial of fulvestrant plus enzalutamide in ER+/HER2– advanced breast cancer

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This clinical trial combined fulvestrant with the anti-androgen enzalutamide in women with metastatic ER+/HER2– breast cancer (BC). Eligible patients were women with ECOG 0–2, ER+/HER2– measurable or evaluable metastatic BC. Prior fulvestrant was allowed. Fulvestrant was administered at 500 mg IM on days 1, 15, 29, and every 4 weeks thereafter. Enzalutamide was given at 160 mg po daily. Fresh tumor biopsies were required at study entry and after 4 weeks of treatment. The primary efficacy endpoint of the trial was the clinical benefit rate at 24 weeks (CBR24). The median age was 61 years (46–87); PS 1 (0–1); median of 4 prior non-hormonal and 3 prior hormonal therapies for metastatic disease. Twelve had prior fulvestrant, and 91% had visceral disease. CBR24 was 25% (7/28 evaluable). Median progression-free survival (PFS) was 8 weeks (95% CI: 2–52). Adverse events were as expected for hormonal therapy. Significant ($p < 0.1$) univariate relationships existed between PFS and ER%, AR%, and *PIK3CA* and/or *PTEN* mutations. Baseline levels of phospho-proteins in the mTOR pathway were more highly expressed in biopsies of patients with shorter PFS. Fulvestrant plus enzalutamide had manageable side effects. The primary endpoint of CBR24 was 25% in heavily pretreated metastatic ER+/HER2– BC. Short PFS was associated with activation of the mTOR pathway, and *PIK3CA* and/or *PTEN* mutations were associated with an increased hazard of progression. Thus, a combination of fulvestrant or other SERD plus AKT/PI3K/mTOR inhibitor with or without AR inhibition warrants investigation in second-line endocrine therapy of metastatic ER+ BC.

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INTRODUCTION

Breast cancer (BC) is a genetically heterogeneous and biologically diverse disease. We currently subdivide BC by estrogen receptor alpha (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2/neu) status, in part because these markers represent important predictive biomarkers that guide treatment with discernible survival benefits. Endocrine therapies, such as tamoxifen, fulvestrant, and aromatase inhibitors (AI), target ER directly or the production of estrogen and play a critical role in the treatment of patients with ER+ disease¹. Androgen receptors (AR) are expressed in most BC, and AR positivity (nuclear staining by immunohistochemistry (IHC) in 10% or more of cells) was observed in 77% of 3093 invasive breast tumors of all subtypes, including 91% of ER+ BC². The predominately nuclear localization of AR by IHC² indicates that it is in the liganded state. In primary ER+ BC compared to patient-matched metastatic disease, AR is commonly maintained in metastases, while ER often decreases³.

The functional role of AR in ER+ BC remains controversial⁴, with confusion arising from the fact that when estradiol is present, androgens decrease ER-mediated proliferation in cell lines and xenograft models⁵. In contrast, in the absence of estrogen, androgens stimulate proliferation, and AR is associated with resistance to tamoxifen and aromatase inhibitors (AI) in ER+ BC^{3,6–10}. Tumors that respond to tamoxifen express similar percent

cells positive for ER and AR protein, as does adjacent uninvolved epithelium¹⁰. However, tamoxifen-resistant breast tumors have a high ratio of percent cells positive for nuclear AR versus ER. A ratio of AR:ER ≥ 2.0 in primary tumors is associated with an over four-fold increased risk for failure while on adjuvant tamoxifen and overall disease-free survival, with an independent effect on risk for relapse beyond ER positivity alone⁹. Similar findings have been reported for patients treated with adjuvant AI therapy^{11–13}.

While AIs effectively block the conversion of androgens to estrogens to decrease ER-stimulated tumor growth, over time, circulating and intra-tumoral androgens can increase as an unintended consequence^{14–17}, resulting in AR activation and resistance to AI therapy. In the absence of estradiol (E2) (as in post-menopausal women on AI therapy) dihydrotestosterone (DHT) (which cannot be aromatized to estrogen) increased proliferation of ER+ cell lines and patient-derived xenografts^{3,9,18,19}. On the other hand, the selective androgen receptor modulator enobosarm decreased tumor growth in preclinical models⁵. A recent trial NCT02007512 using the AI exemestane, with or without enzalutamide, in patients with ER+ advanced/metastatic disease found that high levels of AR and low levels of *ESR1* were associated with the significantly greater benefit of enzalutamide²⁰. These complexities emphasize the importance of clinical context and hormonal milieu when considering AR action in BC²¹. Clearly, AR does influence BC biology, and high AR relative

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to ER levels can serve as an independent predictor of response to anti-estrogen therapies, perhaps by identifying tumors poised to escape ER-directed therapies and switch to survival dependent on androgens and AR. Thus, we postulated that when ER+ BC becomes resistant to ER-targeting therapies, agents targeting AR may provide clinical benefits.

The use of the anti-androgen enzalutamide showed efficacy in women with TNBC²², and it is now under investigation in women with ER+ BC. An extended phase I trial of enzalutamide identified 160 mg/day as the recommended phase 2 dose, and the PK and safety profile in women was similar to that observed in men²³. Enzalutamide is a potent CYP3A4 inducer and reduced the areas under the concentration-time curve (AUC) of anastrozole and exemestane by 80% and ~50%, respectively. However, when combined with fulvestrant, no significant PK interaction or new safety signals were found^{24,25}. Preclinical modeling showed synergistic inhibitory effects of fulvestrant plus enzalutamide on tumor cell growth³. Therefore, we hypothesized that enzalutamide, combined with fulvestrant, would be effective in patients with ER+ AR+ metastatic BC resistant to traditional therapeutic strategies targeting ER or estrogen production (AI therapy). The primary objective of the current trial was to determine CBR24 of the combination of enzalutamide and fulvestrant in metastatic BC originally diagnosed as ER+ disease. The secondary objectives were to confirm the safety profile of the combination, response rate, and progression-free survival (PFS) at 24 weeks. Serial biopsies were obtained during pretreatment and at the end of the fourth week of treatment (hence referred to as week 5) to evaluate the effects of treatment on the tumor and its relationship to clinical outcomes.

RESULTS

Demographics (32 eligible patients evaluable for toxicity)

Of the 32 eligible participants, 28 were evaluable for response (Table 1). The median age was 61 [46–87] years, and the median ECOG PS was 1 [0–2]. Patients were heavily pretreated with a median of 3 [1,9] prior hormonal agents and 4 [0,8] prior non-hormonal therapies. Twelve (37.5%) patients had prior fulvestrant, and 29 (90.6%) had visceral disease.

Treatment-emergent adverse events (AEs) were consistent with what would be expected with hormonal therapy. Fatigue, nausea, and achiness were most common. Cognitive dysfunction described as “difficulty concentrating” was reported in 5 patients and resolved upon completion of treatment. AEs greater than 20% included fatigue (53.1%), nausea (50.0%), vomiting (28.1%), constipation (31.2%), anorexia (28.1%), headache (34.4%), achiness (43.8%), and tumor-associated pain (31.3%). Most AEs were low-grade. G3 toxicity was uncommon and, in most cases, was unrelated to protocol treatment. There were no G4 or G5 toxicities (Table 2).

CBR and overall PFS (28 evaluable patients)

At week 24, 7 (25.0%) (95% CI: 10.7–44.9) participants had stable disease, and no participants had a partial response (Fig. 1a). The median time to progression was 8 weeks (95% CI: 8–20) (Fig. 1b).

Immunohistochemistry for hormone receptors and Ki67

The association between time and each IHC outcome was assessed with univariate linear mixed models. On average, ER Score was 63.3 (95% CI: 24.9–101.6; $p = 0.003$) units lower at week 5 than at baseline, and Ki67 was 10.9 (95% CI: 0.1–21.8; $p = 0.05$) percentage points lower at week 5 than at baseline. No other IHC variable, including PR and glucocorticoid receptor (GR), had a significant difference between baseline and week 5 of treatment (Fig. 1c).

Table 1. Patient characteristics.	
Characteristic	Number (percentage/range)
Consented	38 (6 screens fail)
Eligible and evaluable	32 (100.0%)
Age at the time of consent (years)	
Median (range)	61 (46, 87)
ECOG PS	
Median (range)	1 (0, 2)
Adjuvant chemotherapy	14 (43.8%)
Neoadjuvant chemotherapy	5 (15.6%)
Adjuvant endocrine therapy	20 (62.5%)
Chemotherapy for metastatic disease	15 (46.9%)
Endocrine therapy for metastatic disease	25 (78.1%)
Prior fulvestrant	12 (37.5%)
Prior agents for advanced breast cancer	
Median (range)	7 (2, 15)
Hormonal	
Median (range)	3 (1, 9)
Non-hormonal	
Median (range)	4 (0, 8)
Metastatic sites	
≥3 metastatic sites	17 (53.1%)
Bone	24 (75.0%)
Visceral	29 (90.6%)
Evaluable or measurable disease	28 (87.5%)
AR ≥ 10% Positive (N = 27)	20 (74.1%)
ER ≥ 10% Positive (N = 27)	22 (81.5%)
PR ≥ 10% Positive (N = 27)	12 (44.4%)
AR:ER Ratio ≥ 2 (N = 24)	5 (20.8%)
% Baseline IHC Ki67 (N = 27)	
Median (range)	50 (0, 95)
% Week 5 IHC Ki67 (N = 27)	
Median (range)	40 (3, 95)
ESR1 mutation	11 (34.4%)
P53 (N = 29)	14 (48.3%)
PTEN/PIK3CA (N = 29)	
PIK3CA and PTEN	3 (10.3%)
PIK3CA Only	8 (27.6%)
PTEN Only	3 (10.3%)
Neither	15 (51.7%)

aBC advanced breast cancer, AR androgen receptor, ECOG PS Eastern Cooperative Oncology Group Performance Status, enza enzalutamide, ER+/PgR+ estrogen receptor-positive/progesterone receptor-positive, HER2 human epidermal growth factor receptor 2.

Demographic and clinical risk factors of progression: multivariate Cox proportional hazard survival analysis

Dichotomous AR and ER percent positive at baseline were included in the multivariate PFS model. Due to sample size restrictions, PIK3CA/PTEN and prior fulvestrant were excluded. The hazard of disease progression for participants with AR < 10% positive was 2.46 (95% CI: 0.97–6.22) times the hazard for participants with AR ≥ 10% positive after controlling for ER percent positive ($p = 0.057$). The hazard of disease progression for participants with ER < 10% positive was 4.69 (95% CI: 1.53–14.35) times the hazard for participants with ER ≥ 10% positive, after controlling for AR percent positive ($p = 0.007$) (Table 3, Fig. 1d).

Table 2. Adverse events.

AE	Any grade (N = 32)	G3 related (N = 32)
<i>Constitutional symptoms</i>		
Fatigue	17 (53.1%)	2 (6.2%)
Hot flashes	6 (18.8%)	0
Insomnia	6 (18.8%)	0
Anxiety	4 (12.5%)	0
<i>Gastrointestinal</i>		
Nausea	16 (50%)	0
Vomiting	9 (28.1%)	1 (3.1%)
Diarrhea	7 (21.9%)	0
Constipation	10 (31.2%)	0
Anorexia	9 (28.1%)	0
Dyspepsia	3 (9.4%)	0
<i>Neurologic</i>		
Cognitive disorder	5 (15.6%)	0
Lightheaded	6 (18.8%)	0
Headache	11 (34.4%)	0
<i>Miscellaneous</i>		
Achiness	14 (43.8%)	1 (3.1%)
Itch/Rash	3 (9.4%)	0
UTI	4 (12.5%)	1 (3.1%)
Hair Loss	1 (3.1%)	0
Tumor-associated pain (NR)	10 (31.3%)	0
Back pain (9) and hepatic pain (1) reclassified as tumor-associated pain (NR). Flatulence, malaise, dry mouth, visual changes, stomach cramps, T wave flattening, breast swelling, nails peeling (one each, G1/2). No G4 or G5 toxicities.		

A Kaplan–Meier curve of PFS stratified by AR and ER protein by IHC suggested that those with biopsies with <10% positive cells for both ER and AR had the shortest PFS, and those with ≥10% of both receptors received the longest PFS, albeit sample size was too limited to perform statistical tests (Fig. 1d).

Demographic and clinical risk factors of progression: Univariate survival analysis

Significant ($p < 0.2$) univariate relationships were observed between continuous PFS and the following variables: ER and AR percent positivity by IHC and *PIK3CA* and/or *PTEN* mutations. Specifically, the average hazard for progression was 4.32 (95% CI: 1.46–12.83) times higher for participants with ER < 10% positive compared to participants with ER ≥ 10% positive ($p = 0.008$). This association remained significant when ER was considered continuous ($p = 0.024$). Additionally, the average hazard of progression was 2.28 (95% CI: 0.93–5.62) times higher for participants with AR < 10% positive compared to participants with AR ≥ 10% positive ($p = 0.073$). The hazard of disease progression for participants with AR and/or ER < 10% positive was 2.35 (95% CI: 0.99–5.58; $p = 0.052$) times the hazard for participants with AR and/or ER ≥ 10% positive. Finally, the hazard of disease progression for patients with *PTEN* and/or *PIK3CA* mutated tumor biopsies was 2.02 (95% CI: 0.89–4.56; $p = 0.092$) times the hazard for participants with neither molecular event. This is illustrated in a swimmer plot of PFS, prior fulvestrant, and prior everolimus with activating mutations in the *PIK3CA* or potential loss of function *PTEN* mutations, as well as *ESR1* mutations, indicated (Fig. 1e). No other factors of interest,

including *ESR1* mutations, prior exposure to fulvestrant (38%), everolimus (28%), or cdk4/6 inhibitors (83%) had significant univariate associations with PFS (Table 3, Fig. 1e).

Reverse phase protein array demonstrates activation of the mTOR pathway in tumors from patients in the short PFS group

When comparing phosphorylated proteins in the “Short PFS” defined in all RPPA analyses as PFS ≤ 60 days ($n = 20$) to those who experienced “Long PFS” defined as PFS > 24 weeks (168 days) ($n = 7$), the mTOR pathway had higher baseline expression levels in the tumors of those with Short PFS. According to robust moderated *t*-tests, baseline mTOR S2448 ($p = 0.008$), eNOS/NOSIII S116 ($p = 0.029$), S6RP S240/S244 ($p = 0.031$), eIF4G S118 ($p = 0.037$), and p70S6K T389 ($p = 0.043$) were significantly higher in the Short PFS group compared to the Long PFS group (Fig. 2a and Supplementary Table 1). Similar results were obtained when Short PFS was defined as <24 weeks ($n = 25$). This suggests mTOR activation in the pre-treatment tumor biopsies from patients with a short time to progression (Fig. 2b). However, the differences in the baseline abundance of each mTOR protein were not significantly different between tumors with *PTEN/PIK3CA* mutants versus those without these mutations and were also not associated with prior everolimus.

We also assessed the RPPA data for phospho-proteins that changed pre- versus post-enzalutamide plus fulvestrant in patients with Short versus Long PFS. Proteins differentially altered by treatment in the Short PFS group compared to the Long PFS group included phosphorylated forms of cABL Y245, LKB1 S334, PAK1/PAK2 S199, S204/S192, S197 and S6RP S235, S236, and RB S780, respectively (Fig. 2c and Supplementary Table 2).

Phospho-proteins that correlate with ER or AR at baseline and with the change after treatment

There were 74 (47.1%) proteins that had significant baseline associations with total ER in the RPPA data (Supplementary Table 3a), 72 (97.3%) of which were positively correlated, meaning that patients with lower ER tended to have lower values of correlated proteins and patients with higher ER tended to have higher values of correlated proteins. Additionally, 86 (54.8%) of proteins had a significant association with the change in ER; 85 (98.8%) of which were positively correlated (Supplementary Table 3b). Of note, total ER positively correlated with ERpS118, PRpS190, total AR, ARpS81, as well as total Cyclin D1 and many other interesting phospho-proteins. Many of the same proteins changed along with ER following treatment.

Similarly, 48 (30.6%) proteins had significant baseline associations with AR, and 80 (51.0%) proteins had significant fold change associations with AR. Respectively, 48 (100%) and 79 (98.8%) were positively correlated (Supplementary Table 4a, b). pARS81 correlated with total AR at baseline, as did total Cyclin D1, total ER, and total HER2. These proteins also changed with AR following treatment.

Proteins that correlated with ER and AR were enriched for several pathways (Supplementary Tables 5a, b and 6a, b). Response to estradiol and epithelial cell proliferation corresponded with baseline ER levels and the change in ER with treatment (Supplementary Table 5a, b). Proteins correlated with baseline AR corresponded to hair follicle development, negative regulation of heart valve morphogenesis and mesenchyme morphogenesis, and negative regulation of transcription and translation (Supplementary Table 6a), and some of the same pathways and embryonic development changed with AR with treatment as well (Supplementary Table 6b).

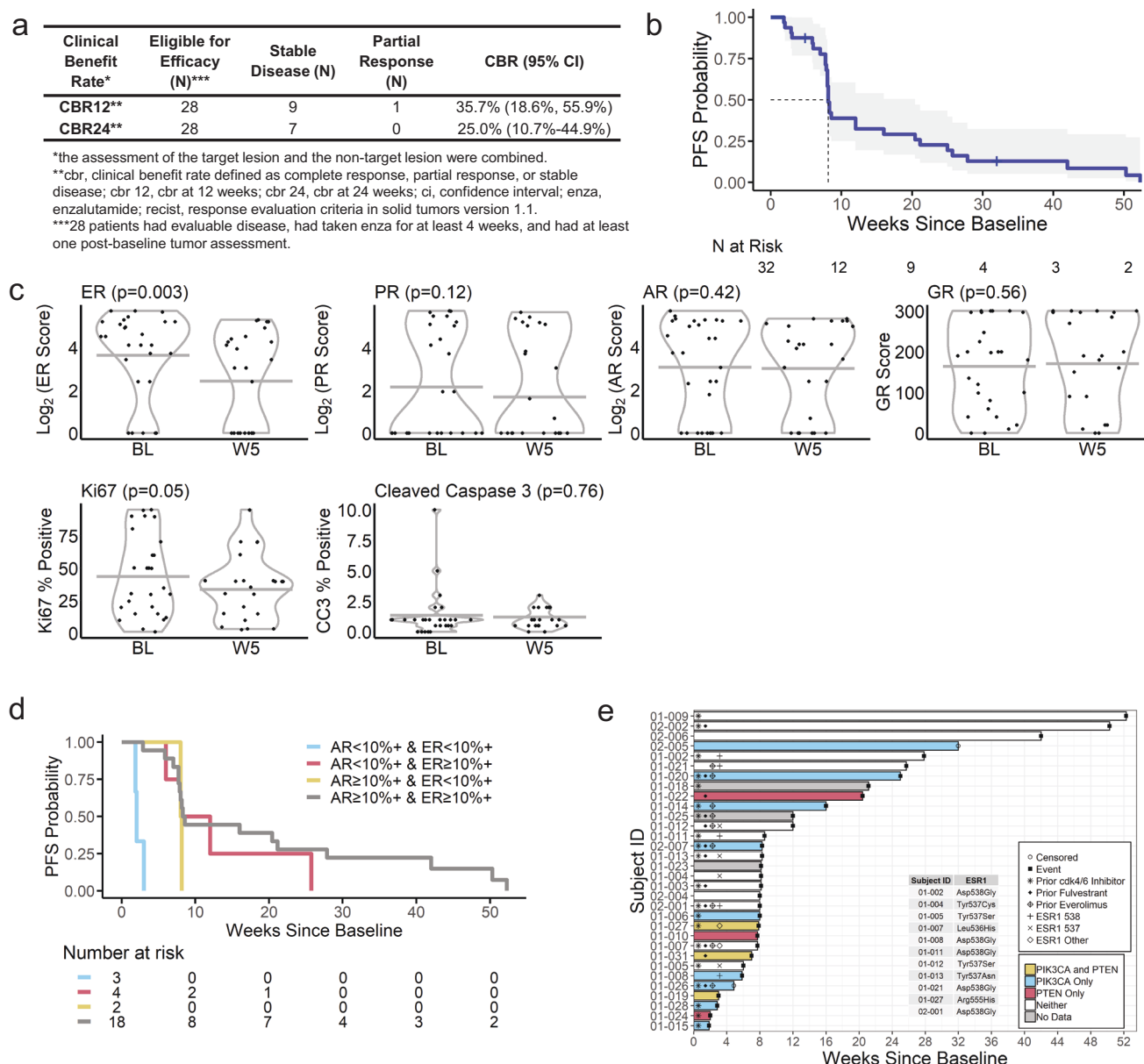


Fig. 1 Efficacy of enzalutamide plus fulvestrant in advanced ER+/HER2– breast cancer patients. **a** Clinical benefit rate at 12 weeks (CBR12) and 24 weeks (CBR24). **b** Kaplan–Meier of progression-free survival (PFS) in all patients treated with enzalutamide plus fulvestrant. Censored times are marked with vertical dashes, and the median time to progression (8 weeks) is noted. **c** Violin plots of steroid hormone receptor IHC quantification ER, AR, PR, GR, as well as Ki67 and Cleaved Caspase 3 (CC3) IHC, are stratified by baseline (BL) and week 5 (W5) of treatment. Plots show the distribution of the observed values with the time-specific minimum and maximums as the lowest and highest gray horizontal lines, respectively. Corresponding *p*-values and black points indicate observed values as assessed with the Wald two-sided test. **d** Kaplan–Meier curve of overall survival probability in patients treated with enzalutamide plus fulvestrant stratified by AR and ER protein expression as determined by immunohistochemistry. **e** Swimmer plot representing each patient's progression-free survival time in weeks. Censored end times are marked with open circles, and participants who experienced an event are marked with black squares. *PIK3CA* and *PTEN* are represented by color, and prior treatments and biomarkers are indicated with symbols.

Plasma metabolomics analyses

Metabolomics analyses of patient plasma revealed baseline and post-treatment differences in acyl-carnitines, amino acid, and purine metabolism in patients with Short versus Long PFS. Given the role of mTOR as a master sensor and regulator of metabolism^{19,26,27}, compared to Long PFS patients, plasma from patients with Short PFS had higher levels of several acyl-carnitines (C10, 12, 12:1, 14:1, 18:2), but a lower level of amino acids (L-arginine, citrulline, glutamate, lysine, methionine) and purine breakdown and deamination products (inosine, xanthine, guanosine) at baseline (Fig. 3a). Following treatment, purine oxidation

products (hypoxanthine, xanthine, 5-hydroxyisourate) and amino acids (asparagine, glutamine, glutamate) were higher in the Short PFS group (Fig. 3b). However, serine, citrulline and the main soluble intracellular antioxidant tripeptidylglutathione, were higher in plasma from Long PFS patients (Fig. 3b). This is relevant considering the role of mTOR in amino acid sensing—especially serine²⁸. Increased consumption of fatty acids and preservation of plasma acyl-carnitine levels were observed following treatment in Long PFS patients. Based on these results, both liver functions, transamination (glutamate, alpha-glutamate), and glutathione metabolism are elevated in the plasma of the Short PFS group.

Table 3. Univariate and multivariate analyses.

Time to disease progression			
	Hazard ratio	CI	p
<i>Univariate predictors</i>			
AR < 10% Positive (N = 27)	2.28	0.93–5.62	0.073
ER < 10% Positive (N = 27)	4.32	1.46–12.83	0.008
AR and/or ER < 10% Positive (N = 27)	2.35	0.99–5.58	0.052
PR < 10% Positive (N = 27)	0.73	0.32–1.67	0.452
ESR1 mutation (N = 32)	1.47	0.68–3.19	0.329
Ki67 decreased ^a (N = 21)	1.79	0.70–4.59	0.225
PTEN and/or PIK3CA (N = 29)	2.02	0.89–4.56	0.092
<i>Multivariate predictors (N = 27)</i>			
AR < 10% Positive	2.46	0.97–6.22	0.057
ER < 10% Positive	4.69	1.53–14.35	0.007

Select univariate associations with progression are presented. The reference level for “AR and ER < 10% Positive” are participants with AR ≥ 10% positive and/or ER ≥ 10% positive. The reference level for “PTEN and/or PIK3CA” are patients with neither loss. All other univariate associations of interest were non-significant predictors of progression, with *p*-values greater than 0.2.

^aKi67 decreased refers to patients who had a decrease in Ki67 between baseline and week 5. The reference group is patients who had an increase or no change in Ki67 between baseline and week 5.

DISCUSSION

The current phase II clinical trial investigated the combination of fulvestrant with enzalutamide in a heavily pretreated population of women with metastatic ER+/HER2– BC. The regimen achieved CBR24 in 25% of the patients, with generally low-grade toxicity with no new safety signals. It is notable that 29% (2 of 7) patients in the Long PFS (PFS > 24 weeks) group had prior exposure to fulvestrant. Analyses of tumor biopsies demonstrated that patients with ≥10% positive cells for ER or AR by IHC had longer PFS than patients with ER and AR < 10% positive. Metastatic disease evolves under the selective pressure of treatments and can lose expression of ER^{29,30}, and this was observed in 13% of the patients in this study. In primary tumors versus patient-matched metastases, we previously found that nuclear AR was often maintained even when ER was reduced or absent³. These data emphasize the need to consider fresh biopsy to confirm the presence of the therapeutic targets of interest.

Patients with Short PFS (PFS ≤ 60 days) had increased mTOR pathway activation in the pre-treatment biopsies compared to patients with PFS > 24 weeks. Interestingly, these findings were supported by plasma metabolomics data, showing significantly lower levels of multiple amino acids in patients with Short PFS (<24 weeks) compared to Long PFS (>24 weeks)—an observation that would be consistent with mTOR activation via sensing of systemic amino acid depletion³¹. In addition, patients with Long PFS are characterized by preservation of circulating levels of lipid oxidation markers (acyl-carnitines) compared to patients with Short PFS, suggestive of preserved mitochondrial function³², in the former group following treatment with fulvestrant with enzalutamide. This suggests potential utility for adding an mTOR inhibitor such as everolimus to enzalutamide, particularly for patients with activated mTOR or PIK3CA/PTEN mutations, to potentially enhance response rate as has been suggested previously by preclinical and clinical studies^{19,33}. PIK3CA and PTEN mutations are frequent in metastatic ER+ BC^{34,35}. In this study, we find that PIK3CA activating and or PTEN inactivating mutations were significantly associated with a greater hazard of PFS.

Recently, a randomized phase II trial comparing the aromatase inhibitor exemestane alone (25 mg daily) to exemestane (50 mg daily) plus enzalutamide was completed²⁰. Overall, 247 patients with ER+ BC were randomized into two cohorts (one with no prior endocrine therapy for metastatic disease and the other with one prior endocrine therapy in that setting). In this study, only the cohort without prior endocrine therapy had a numerical advantage in PFS for the addition of enzalutamide in patients with high AR (above the median), particularly in combination with low (below median) levels of *ESR1* mRNA in their primary tumors. These patients had a reduced risk of progression or death compared to the control arm [HR, 0.24 (95% CI: 0.10–0.60); *p* = 0.001, with the median PFS extended by 10.2 months (from 3.8 in the control arm to 14.0 months in the enzalutamide arm)²⁰. These data may suggest that primary tumors with higher-than-average AR but lower-than-average *ESR1* are poised to become resistant to AI therapy and may benefit from anti-androgen therapy. This is in line with the high AR to ER percent cells positive data summarized in the introduction³⁶. Interestingly, enobosarm, a selective androgen receptor modulator (SARM) with both agonist and antagonist activity depending on tissue type, showed activity in preclinical^{5,37} and clinical studies³⁸. In women with endocrine-sensitive ER+ BC on adjuvant endocrine therapy (ET) for at least 3 years or who responded to the most recent ET for metastatic disease for at least 6 months, CBR24 was 32% for those treated with enobosarm (9 mg daily), and 29% for those in the 18 mg cohort. Responses were observed in patients with AR+ BC (>10% nuclear AR staining)³⁸. Another recent trial examined the efficacy and safety of enzalutamide with trastuzumab in patients with HER2+/AR+ locally advanced or metastatic BC in a single-arm phase II study in heavily pretreated patients with advanced HER2+ AR+ BC and observed a 24% CBR24³⁹.

A major strength of NCT02953860 includes paired tumor biopsies pre-treatment and on-treatment to assess mutations, phospho-protein expression, and associations with PFS. We find that 7 (25%) of patients reached CBR24, including 2 who were progressing on prior fulvestrant, suggesting possible benefit for this subset. The study lends insight into the pathways that might be of importance regarding resistance, such as mTOR activation. Other than ER ≥ 10%, AR ≥ 10%, and the absence of *PTEN* or *PIK3CA* mutations, there were no clear indicators of response. Limitations include a heavily pretreated population with substantial heterogeneity of prior treatment. The sample size is limited; thus, all correlations are by nature exploratory. Future research must be conducted to evaluate the reproducibility of the results. Regarding the interpretation of the plasma metabolomics, we acknowledge that system-wide metabolomics analyses cannot distinguish between tumor versus organ response to drugs; however, the differences between the Short and Long PFS groups are intriguing. These analyses were performed at a steady state in vivo, and while more relevant than ex vivo studies, they are confounded by factors such as dietary or other physiological effects of the treatment, depending on patient-to-patient heterogeneity in response to the regimen.

If mTOR activation is confirmed as a potential mechanism of de novo resistance to enzalutamide, the addition of mTOR inhibitors to ER/AR blockade could be a feasible trial since combinations of enzalutamide and AR inhibitors with mTOR or PI3K inhibitors have proven clinical safety^{40,41}. We recently completed accrual to a companion phase II trial (NCT02955394) of fulvestrant with or without enzalutamide in the neoadjuvant setting in women with T2 or greater ER+/HER2– BC, also with serial biopsies. The neoadjuvant trial will not have the confounding factor of the various prior treatments inherent to this metastatic trial. In conclusion, the present phase II study demonstrates that enzalutamide can be given safely in combination with fulvestrant for ER+ metastatic disease and that this combination warrants additional investigation. Future trials could be done in second-line

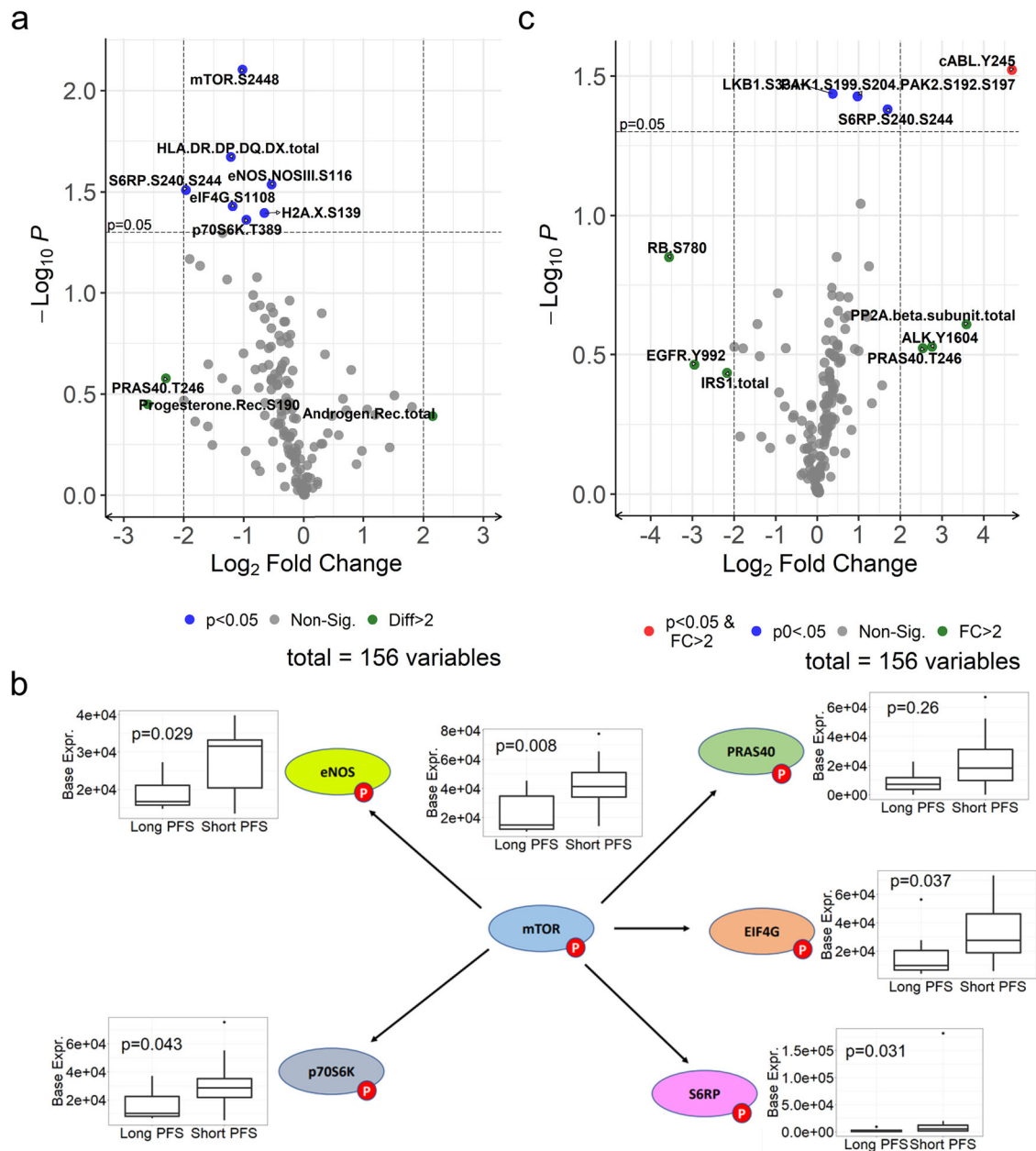


Fig. 2 RPPA analysis of frozen core biopsies from tumors at baseline and following enzalutamide plus fulvestrant. **a** Volcano plot of differences in baseline detection across the Long and Short PFS groups. **b** Differentially expressed phospho-proteins in the mTOR signaling pathway in Long PFS versus Short PFS at baseline. The lower and upper bounds of the boxes represent the first (Q1) and third (Q3) quartiles, respectively, which cover the interquartile range (IQR). The horizontal line within each box represents the median. The whiskers extending vertically out from the boxes are computed as $Q1 - 1.5 \times IQR$ and $Q3 + 1.5 \times IQR$. Outliers are data points outside of this range and are indicated with dots. **c** Volcano plot of differences in fold change detection across Long and Short PFS groups. All statistical analyses were performed with the Bayes log₂ two-sided moderated *t*-test.

endocrine therapy (progressing after AI and cdk4/6 inhibition) and in PI3K wild-type tumors to evaluate fulvestrant plus everolimus with or without an AR signaling blocker.

METHODS

Study design and treatments

NCT02953860 (COMIRB 16–1001) was an open-label, non-randomized trial combining fulvestrant plus enzalutamide. All patients received fulvestrant 500 mg IM on days 1, 15, 29, and then every 4 weeks plus enzalutamide 160 mg po daily until disease progression or unacceptable toxicity. Pre- or peri-

menopausal women received concurrent ovarian suppression with a gonadotropin-releasing hormone agonist.

Study approval and ethics

The study was conducted at the University of Colorado and the University of Tennessee. The study protocol and its amendments were approved by the respective Institutional Review Boards, including the Colorado Multiple Institutional Review Board (COMIRB), the Office of Human Research Oversight (OHRO), and the University of Tennessee Institutional Review Board (UTHSC IRB). All patients provided written informed consent prior to participating in the study. The study was conducted under the

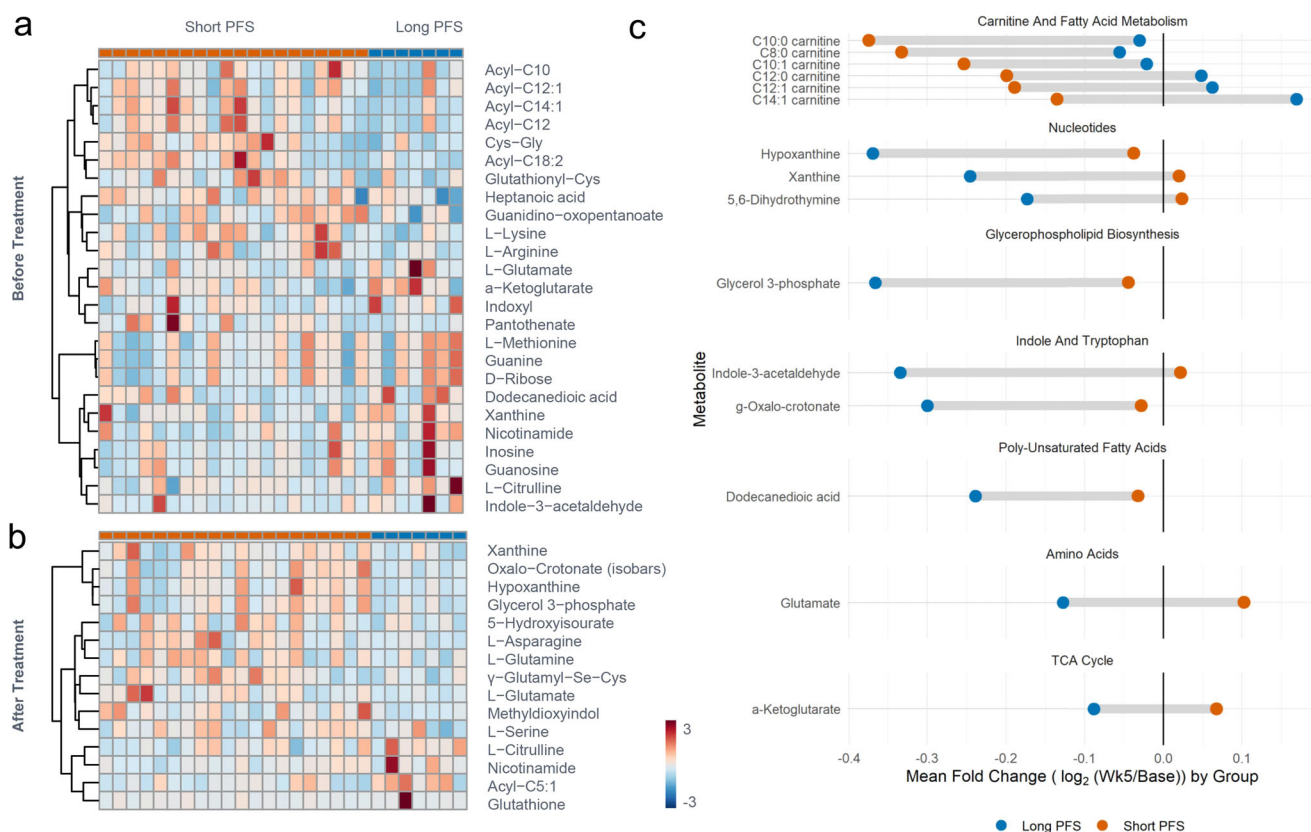


Fig. 3 Analysis of plasma metabolomics performed via UHPLC-MS at baseline and after treatment. Metabolites with significant differences between Long and Short PFS are presented in heatmaps **a** at baseline and **b** after treatment. **c** The mean fold changes with treatment are presented for metabolites with significant differences between the Long and Short PFS groups.

principles of the World Medical Association, the Declaration of Helsinki, and the Good Clinical Practice guidelines of the International Conference on Harmonization. The study did not require an Investigational New Drug Application. Drug support (enzalutamide) was provided by Astellas and Pfizer as part of this investigator-sponsored research study.

Study population

Eligible patients were women ≥ 18 years of age with adequate organ and bone marrow function and an ECOG performance score (PS) of 2 or less. All had metastatic BC determined to be ER-positive and HER2-negative. Prior anti-androgen treatment was not allowed. Prior fulvestrant was allowed if the treating physician felt that retreatment with fulvestrant was clinically indicated. The measurable or evaluable disease was required. Men were excluded due to potential confounding from androgenic stimuli. Central nervous system (CNS) metastases or a history of seizures were exclusionary due to the toxicity profile of enzalutamide. Determination of AR expression was not a requirement as it was expected that $\sim 90\%$ of tumors would stain for AR, and the assay has not yet been validated for clinical decision-making. Concomitant medications with substantial pharmacokinetic (PK) interaction with enzalutamide were avoided.

Safety and antitumor assessment

All patients who received at least one dose of enzalutamide were assessed for safety biweekly for the first 4 weeks, then every 4 weeks until 30 days after the last dose of enzalutamide or prior to the initiation of a new treatment, whichever occurred first. Safety and tolerability were determined by assessment of AEs, physical examinations, ECOG PS, vital signs, and laboratory tests.

The severity of abnormal laboratory values and AEs were classified using the National Cancer Institute Common Terminology Criteria for AEs (CTCAE), version 4.03. SAEs were also evaluated by Astellas Pharma Global Development—United States. A monthly teleconference was held among the institutional investigators to review patients and AEs. An institutional Data Safety and Monitoring Committee at the University of Colorado also had oversight for monitoring.

Radiographic assessments of disease status were performed at baseline and every 8 weeks thereafter. Tumor responses were defined using Response Evaluation Criteria in Solid Tumors (RECIST 1.1) version 1.1 criteria. Patients evaluable for response, PFS, and CBR had a follow-up scan or withdrew from the study because of toxicity or clinical progression.

Tissue acquisition

Fresh tumor biopsies (punch biopsies for skin lesions or core needle biopsies for other sites) were required at study entry (baseline), and after 4 weeks on therapy (when both fulvestrant and enzalutamide were likely at steady state concentrations) and these were termed “WK5” biopsy. Archival tissue from the primary or prior biopsies of metastatic disease was obtained if available. A tumor biopsy at the time of progression was requested but is optional. Lithium-heparin (LiHep) plasma samples were obtained at the same intervals. After collection LiHep vacutainers were centrifuged for 20 min ($600\times g$), separated plasma was transferred to a 15 mL conical tube, centrifuged a second time for 15 min ($1500\times g$), aliquoted into 500 μ L aliquots, and stored at -80°C until analysis.

Statistical analysis: sample size and data analysis considerations

CBR24 was used as the primary endpoint for sample size. Assuming the undesirable rate of 10% and the desired rate of 30%, a sample size of 24 provides 89% power to detect this 25% rate difference using an exact binomial test with a one-sided alpha of 0.085. Due to the exploratory nature of biomarker analyses, the type I error rate for all analyses was not adjusted for exploring multiple biomarkers. Of the 38 participants who consented to the clinical trial, 32 (84%) were eligible and evaluable, and 28 (74%) were considered evaluable for the CBR endpoint because they had at least one post-baseline tumor assessment. The 4 patients who withdrew early did not withdraw due to disease progression or for reasons of toxicity. Three (9.3%) participants had a fresh biopsy that stained negative for both AR and ER; they were thus excluded from the AR:ER ratio analysis. All missing observations were eliminated from the respective univariate analyses (Table 1).

Clinical benefit rate and overall PFS

The clinical benefit rate was defined as the percent of patients with a response or stable disease by RECIST 1.1 criteria at the week 24 assessment (CBR24). A Kaplan–Meier survival curve was used to determine the median time to progression.

Demographic and clinical risk factors of progression: univariate survival analysis

To identify the demographic and clinical risk factors of progression, the univariate relationships between PFS and each of the following risk factors were assessed with Cox Proportional Hazard models (CoxPH): age, ECOG PS, metastatic sites, bone metastatic sites (≥ 1 vs. 0; all vs. some/no sites), baseline and change in IHC Ki67, *ESR1*, and *PIK3CA/PTEN* mutation status, AR, ER, PR (score; $\geq 10\%$ vs. $<10\%$ positive, continuous % positive), and the AR:ER ratio (<2 vs. ≥ 2). Additionally, the following lines of treatment were assessed: adjuvant chemotherapy, neoadjuvant chemotherapy, adjuvant endocrine therapy, chemotherapy for metastatic disease, endocrine therapy for metastatic disease, prior fulvestrant, the number of prior agents, the number of hormonal prior agents, and the number of non-hormonal prior agents.

Multivariate Cox proportional hazard survival analysis

To assess the multivariate associations between risk factors and PFS, prior fulvestrant and all factors that had moderately significant ($p < 0.2$) univariate associations with PFS were included in a multivariate CoxPH model. Analyses were performed using R version 4.0.2.

Mutation analyses methods

Core needle biopsies were acquired from patients who gave informed written consent, with ER⁺/HER2[−] measurable or evaluable MBC without CNS disease. Formalin-fixed paraffin-embedded (FFPE) sections were analyzed for mutations in *ESR1* exon 8 and 67 other gene hotspots in genes frequently altered in cancer using a modified Archer VariantPlex Solid Tumor Assay through the CMOCO Laboratory (Department of Pathology, University of Colorado, Aurora, CO). The majority (60%) of biopsies were from the liver. The other sites included lymph nodes (16%), other soft tissue (9%), bone (9%), skin (3%), and breast (3%). Patients' original primary tumors consisted of invasive ductal carcinomas (63%), invasive lobular carcinoma (18.5%) and invasive mammary carcinoma (14.8%), and unknown (3.7%).

Immunohistochemistry

IHC was performed for ER (0.36 $\mu\text{g/mL}$ clone 1D5 #M7047, Agilent/Dako, Santa Clara, CA), PR (0.5 $\mu\text{g/mL}$ clone PgR 1294 #M3568,

Agilent/Dako, Santa Clara, CA), AR (2.15 $\mu\text{g/mL}$ clone 441 #M3562, Agilent/Dako, Santa Clara, CA), and GR (1:200 #3660 Cell Signaling Technologies, Danvers, MA) as well as Ki67 (0.2 $\mu\text{g/mL}$ clone MIB-1 #M7240, Agilent/Dako, Santa Clara, CA) and cleaved caspase 3 (1:400 #9661, Cell Signaling Technologies, Danvers, MA) as described previously⁷. Briefly, biopsies from patients were formalin-fixed, paraffin-embedded, and 5 μm sections were heat immobilized onto charged slides. Slides were deparaffinized and rehydrated in a series of xylenes, and ethanol and antigens were heat retrieved using either citrate pH 6.0 (PR, AR, GR, Ki67) or 10 mM Tris/1 mM EDTA pH 9.0 buffer (ER, cleaved caspase 3) before probing with primary antibodies. Antibodies were detected using either the Vectastain Elite ABC Universal kit #PK-7200 (ER, PR, AR, Ki67) or the ImmPRESS Goat Anti-Rabbit IgG Polymer kit #MP-7451 (GR, cleaved caspase 3) from Vector Laboratories (Burlingame, CA) followed by DAB detection and counterstained with dilute hematoxylin.

Reverse-phase protein microarray (RPPA)

Enriched epithelial cell subpopulations were isolated from 8 μm cryosections ($>95\%$ purity) using an Arcturus Pixcell Ile Laser Capture Microdissection system (Arcturus, Mountain View, CA, USA) as described⁴². Approximately 10,000 epithelial cells were captured for each sample. Microdissected material was lysed in extraction buffer composed of 1:1 Tissue Protein Extraction Reagent (TPER; ThermoFisher, Waltham, MA, USA) and 2 $\times$ SDS-PAGE Sample Buffer (ThermoFisher) plus 2.5% beta-mercaptoethanol (BME) at a concentration of approximately 500–600 cells per 1 μL . Samples were heated at 100 $^{\circ}\text{C}$ for 5 min, briefly centrifuged and stored at -20°C until printed. Lysates were printed in triplicate spots (approx. 10 nL per spot) onto nitrocellulose coated slides (Grace Biolabs, Bend, OR, USA) using a Quanterix 2470 Arrayer (Quanterix, Billerica, MA, USA). Standard curves of control cell lysates were also included for quality assurance purposes⁴³. Antibodies used on the arrays were validated before use⁴⁴. Immunostaining was performed as previously described⁴⁵. Each slide was probed with one primary antibody targeting each of the 158 proteins of interest (Supplementary Table 7). Biotinylated goat anti-rabbit (1:7500, Vector Laboratories Inc, Burlingame, CA) and rabbit anti-mouse (1:10, DakoCytomation, Carpinteria, CA, USA) IgG were used as secondary antibodies. Signal amplification was performed using a tyramide-based avidin/biotin amplification system (DakoCytomation, Carpinteria, CA, USA) followed by streptavidin-conjugated IRDye 680 (LI-COR, Lincoln, NE, USA) for visualization. Total protein was measured using Sypro Ruby protein blot staining per manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Images were acquired using a Tecan PowerScanner (Tecan, Männedorf, Switzerland) and analyzed with MicroVigene Software Version 5.6. (Vigenetech, Carlisle, MA, USA). The final results represent negative control-subtracted and total protein normalized relative intensity values for each endpoint within a given patient sample.

To assess differences in phospho-protein abundance in pre-treatment tumor biopsies from patients who experienced "Short PFS" (defined in all RPPA analyses as PFS ≤ 60 days) compared to those who experienced "Long PFS" (PFS > 24 weeks), robust moderated t-tests by response were performed on the log₂-transformed baseline abundance of each protein. An empirical Bayes method was employed to shrink sample variances toward a pooled estimate, allowing for a powerful and stable inference to detect significant differences in baseline expression between the Long PFS and Short PFS groups. The analysis was then repeated to compare differences in the change in abundance between baseline and the beginning of week 5 of treatment across PFS groups. Proteins in the mTOR pathway with significant baseline differences between "Long PFS" and "Short PFS" were identified and analyzed with previously described moderated t-tests to

compare patients with *PTEN* and/or *PIK3CA* mutations (*PTEN/PIK3CA* mutants) to *PTEN/PIK3CA* wildtypes at baseline. Baseline and fold change associations between each RPPA phosphoprotein, AR, and ER were then assessed. Each log₂ autoscaled protein and AR were separately assessed using cell means linear regression models with baseline AR or ER as the outcome and the baseline protein of interest as the primary predictor. Each model was adjusted for post-treatment measurements. The association between the fold change of each protein and the fold change of AR or ER was similarly assessed, with the change in AR or ER as the outcome and the change in the protein of interest as the predictor.

We then evaluated whether proteins associated with AR and ER at baseline and with the change with treatment were enriched relative to all analyzed proteins. Proteins were first annotated with the biomaRt R package⁴⁶, and proteins without annotations (18%) were eliminated. Candidate proteins were identified separately for each analysis (AR baseline, ER baseline, AR fold change, ER fold change) based on significance ($p < 0.05$). Gene Ontology (GO) enrichment analysis was then performed using the topGO R package⁴⁷. Fisher's exact tests with >2 proteins per node were used to test for enrichment.

Metabolomics analyses. Plasma metabolomics analyses were performed via UHPLC-MS (Vanquish-QExactive, Thermo Fisher), as previously described⁴⁸. Briefly, plasma (20 μ l) was extracted in 980 μ l of methanol:acetonitrile:water (5:3:2, v/v/v). After vortexing at 4 °C for 30 min, extracts were separated from the protein pellet by centrifugation for 10 min at 18,000 g at 4 °C and stored at -80 °C until analysis. Analyses were performed using a Vanquish UHPLC coupled online to a Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Samples were analyzed using a 5 min gradient as described^{48–50}. Solvents were supplemented with 0.1% formic acid for positive mode runs and 1 mM ammonium acetate for negative mode runs. MS acquisition, data analysis, and elaboration were performed as described^{48–50}.

Baseline and week 5 Metabolomics data analysis was performed via MetaboAnalyst 5.0⁵¹ by comparing autoscale normalized data for "Short PFS" and "Long PFS" groups at baseline and after treatment. In addition, Log₂-transformed autoscaled metabolomic data were analyzed analogously to RPPA data to compare patients who experienced "Short PFS" (defined for all metabolomic analyses as PFS < 24 weeks) with those who experienced "Long PFS" (PFS > 24 weeks) with the change following treatment.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The laboratory data free of protected health information generated in this study are available upon request to the corresponding author.

CODE AVAILABILITY

Data cleaning and analyses were performed in R version 4.0.2 and Bioconductor. Code generated to analyze laboratory datasets free of protected health information is available upon reasonable request to the corresponding author.

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Conceptualization: A.D.E. and J.K.R. Methodology: N.S.S., S.S., A.D., R.I.G., and J.D.W. Funding acquisition: A.D.E. and J.K.R. Patient enrollment: G.A.V., V.F.B., P.K., J.R.D., E.S., A.A., J.M., and G.C. Data curation: A.W.S., K.L.Z., A.vB., and D.G. Formal analysis: A.W.S., A.D., Y.Z., R.I.G., J.D.W., and D.G. Data visualization: N.S.S., A.W.S., L.S.C., and A.A. Project administration: T.M. and G.C. Writing: A.D.E., A.W.S., E.F.P., and J.K.R. Review and editing: A.D.E., N.S.S., L.S.C., G.A.V., V.F.B., P.K., J.R.D., E.S., A.A., J.M., G.C., and D.G. Supervision: A.D.E., A.vB., E.F.P., D.G., and J.K.R.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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