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Breast cancer, androgen receptor, estrogen receptor, growth factors, enzalutamide, endocrine resistance, targeted therapy

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 *in vitro* and results of a Phase 1 study (NCT01597193) on pharma-cokinetics 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 *in vivo*. Here we report on the two trials in ER+ breast cancer one completed enrollment (the trial for women with persistent metastatic ER+ breast cancer and the other (a neoadjuvant trial) with fulvestrant alone compared to fulvestrant plus enzalutamide just finishing up.

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1. 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. In breast cancers, the androgen receptor (AR) is more widely expressed than estrogen receptor alpha (ER) or the progesterone receptor (PR), which are used as therapeutic targets and biomarkers, suggesting a potential role for AR in BC. We examined the primary tumors of women treated with tamoxifen or aromatase inhibitor therapy and found that a higher AR to ER protein ratio correlates with worse response to the anti-estrogen tamoxifen (Cochrane DR et al 2014). In this DOD Clinical Translational Grant we utilize a new-generation AR inhibitor, enzalutamide, which impairs nuclear localization of AR, to explore the function of AR in models of the three main subtypes of breast cancer (ER positive, ER negative and Her2+). This is a very different mode of action than previous generation anti-androgens such as bicalutamide (Casodex), a competitive inhibitor of endogenous androgens that allows ligand-mediated nuclear localization of AR. Enzalutamide has shown success in the clinic in patients with late stage prostate cancer refractory to bicalutamide and is now FDA approved as a prostate cancer therapy. The research in this proposal seeks to determine whether inhibition of AR with enzalutamide will be effective in breast cancer and utilize preclinical models to determine if and how it should be combined with currently used standard of care treatments in the three main types of breast cancer, with the primary objectives of guiding the design of future clinical trials with enzalutamide. The clinical portion of this grant serves to conduct a neoadjuvant trial to study fulvestrant (standard of care) to fulvestrant plus enzalutamide and another trial for women with metastatic ER+ breast cancer treating with fulvestrant plus enzalutamide and to obtain serial biopsies and perform molecular analyses to identify changes in the pre-versus post treatment biopsies in concert with the overall clinical analysis of the efficacy of enzalutamide in ER+ breast cancer. The preclinical portion of the grant is over, but Dr. Richer's lab and the molecular pathology core are performing the final assays on biopsies from neoadjuvant clinical trial. A manuscript on the trial in metastatic breast cancer is under review and is included in the appendix of this report.

2. KEYWORDS: Breast cancer, androgen receptor, estrogen receptor, growth factors, enzalutamide, endocrine resistance, targeted therapy.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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 has been submitted and is under review. Our last goal is to finish analyzing gene expression, RPPA, metabolomics from patient plasma and multiplex analysis of tumor and immune cells from the neoadjuvant 16-1042 trial with one arm being fulvestrant alone and the other fulvestrant plus enzalutamide. The clinical database for that trial at the 3 sites is being locked down now and then we will compile those results and the laboratory analyses and submit the second clinical trial paper for peer review.

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.
Task 2. Test enza in three different tamoxifen resistance models *in vitro.*
Task 3. Test enzalutamide in combination with Her2 directed therapy in ER+ and ER- Her2+ models
Task 4. Examine enzalutamide in combination with an mTOR inhibitor (Afinitor/everolimus)
Task 5. In true 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*.).

What was accomplished under these goals?

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.
- Since the last annual report, we publish that AR regulates TGFbeta 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. Accrual was delayed at all participating institutions during the COVID-19 pandemic and activities in the research laboratories were shut down. **However, accrual and study treatment for both trials are now complete.**

Clinical Aim 4: 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. 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.

Remaining work to be done for 16-1042:

- On 16-1042, we will have 55 patients with matched samples
- Analyze RPPA on baseline, week 4 and surgical tissues. Analyze all with respect to PEPI scores, with respect to changes resulting from treatment. Half of the data for this trial was obtained from Dr. Petricoin's laboratory in September and half is yet to come. We are preparing a manuscript on this trial right now and it will definitely include this interesting data.
- Analyze gene expression analysis (Biospyder). We just obtained this data and will now do the bioinformatics to determine how this data relates to PEPI score and changes resulting from treatment.
- Analyze plasma assays of metabolites. The samples have been sent to our metabolomics core and are in the que for the mass spectroscopy in mid-August.
- Complete NanoString and analyze data.
- Preparation of the 16-1042 trial manuscript has started and Drs. Richer and Elias and the statisticians have been unblinded to complete the analyses and finish the publication. The manuscript will be submitted to Clinical Cancer Research or Journal of Clinical Oncology.
- We requested publication charges to carry forward into this no cost extension and these may be up to $6,000 each for the two manuscripts (one for each trial), so we request $12K total.

We just (end of July) were granted a 6 month no-cost extension for both awards to carryforward the remaining monies for W81XWH-13-1-0091 (Elias) to 02/14/2023 in order to complete the molecular characterization of the tissues and to pay the publication charges on the last two manuscripts on the two trials. We also requested to continue salary support for Dr. Richer, Nicole Spoelstra in her lab (doing all IHC) and Drs. Dexiang Gao and Alyse Staley who are doing the statistical analyses of all of the data. We also requested a small amount of salary support for Tessa McSpadden to continue to manage the clinical trial follow up, Stephanie Hill to lock the clinical database at all 3 sites, and Tiffany Cull to continue regulatory management of the two protocols.

Clinical Aim 5: Protocol 16-1001: fulvestrant plus enzalutamide in ER+/AR+/Her2- metastatic breast cancer (with serial biopsies) was activated for accrual on 06/29/2017 at the University of Colorado. This trial was completed in Nov of 2021. A phase II protocol of fulvestrant plus enzalutamide in AR+/ER+/Her2- metastatic breast cancer was approved by Medivation and Astellas, by COMIRB, and also by HRPO. Enzalutamide drug supply was secured, and the contract signed. Fulvestrant was given at standard doses (at 500 mg IM monthly following a loading dose) and combined with full dose enzalutamide at 160 mg po daily as per the completed phase I trial. The phase I trial demonstrated that there was no significant pharmacologic interaction between the two drugs, and that they could be safely combined with no emerging toxicities. The hypothesis of this single arm phase II trial was that enzalutamide plus fulvestrant, by blocking both AR and ER signaling, would synergize in patients with ER+/AR+ breast cancer. Objectives were 1) to determine the PFS and clinical benefit rate of the combination; 2) to confirm the safety profile of the combination; and 3) to obtain serial biopsies of breast cancer pretreatment, ~4 weeks into treatment, and at time of tumor progression (n = 24 patients with serial tumor biopsies). 38 subjects signed consent. Six subjects screen-failed and 32 subjects were enrolled. This trial is now closed to accrual. The clinical database has been cleaned and locked. 22 treated subjects have matched tumor biopsy tissues at baseline and at 4 weeks.

The trial for metastatic breast cancer 16-1001 completed and the data completely analyzed. We presented posters at the San Antonio BC Conference (2021) and AACR (2022). We assessed percent tumor in FFPE specimens from both trials to prep for targeted mutational analysis for the 3 most common mutations in ER and the mutation in AR (reported in enzalutamide resistance in prostate cancer in AR) was performed using a modified version of the Archer VariantPlex Solid Tumor assay (ArcherDx, Boulder CO) to examine point mutations and insertions/deletions in 69 commonly mutated genes with full exonic coverage for some genes and select exon and hotspot coverage for others. We published a paper with some of the pretreatment data in early 2021/2022: **PMID: 33184106 and PMID: 35538119 in Cancer Research and NPJ Breast Cancer respectively**. In addition, from frozen sections we utilized for RPPA laser captured to enrich for tumor in laboratory of Dr. Chip Petricoin. Dr. Richer is coordinating the molecular analyses with Dr. Petricoin's team

and Alyse Staley for the two trials through our Pathology core. We submitted the manuscript on the results of that trial that is under review in NPJ Breast cancer as of July,2022 (manuscript attached in appendix).

What opportunities for training and professional development has the project provided?

Graduate student that worked on this project, **Valerie Barton** obtained an NIH NRSA F31 on work that stemmed from this grant in Dr. Richer's laboratory. She defended her dissertation and obtained her doctorate from the University of Colorado Cancer Biology Graduate Program April 2016.

Postdoctoral fellows Drs. Nicholas D'Amato and Michael Gordon also completed their tenures in the lab and have taken positions at AstraZeneca and Abbvie respectively.

Nicole Spoelstra, professional research assistant has been integral to this project and performed all of the IHC on the clinical specimens the passes them to pathologist Sharon Sams to read in a blinded fashion. Nicole Spoelstra also performed the TSA Opal mutltiplex Polaris staining for the tumor infiltrating lymphocytes and learned this technique at a week-long workshop in Boston when it first started. She has now performed multiplex IHC for immune cells on all the samples from the metastatic trial and those for the neoadjuvant trial are underway. She is author on all of the manuscripts that came out of this grant with the most recent being PMID: 35538119 in 2022 and the paper on the results of the 16-1001 trial under review.

Dr. Michelle M Williams has been working on preclinical aspects of AR in the ESR1 mutant ER+ disease as a postdoctoral fellow She was first author on Williams MM et al**.** Steroid hormone receptor and infiltrating immune cell status reveals therapeutic vulnerabilities of ESR1 mutant breast cancer. CANCER RES 2021 PMID: 33184106, which contained information about the pretreatment biopsies of metastatic disease from the 16-1001 trial. She is supported on our Cancer Biology T32 and obtained an F32 NCI Fellowship on a different topic. Dr. Williams has now obtained an NCI F99/K00.

Dr. Jessica Christenson, a postdoctoral fellow in the Richer lab, has been on numerous publications emanating from this grant. She now is an instructor and has a MetaVivor Career Development grant. She presented her work on AR in breast cancer lung metastases at the 7 th Annual Metastatic Breast Cancer Research Conference, September 7-9, 2022. EACR-AACR Basic and Translational Research Conference: Tumor Microenvironment, 2020, Lisbon, Portugal Selected poster spotlight speaker: *An activated, pro-tumor lung microenvironment promotes the outgrowth of breast cancer lung metastases, and the* Metastasis Research Society 17th Biennial Congress and Young Investigator Meeting, 2018, Princeton, NJ Selected speaker and poster: *An activated, pro-tumor lung microenvironment promotes the outgrowth of breast cancer lung metastases through lung-specific expression of the androgen receptor.*

Toru Hanamura, MD (a breast surgeon visiting the Richer lab from Japan) worked on AR-regulated secreted factors produced by TNBC and ER+ breast cancer.

Dr. Crump, new postdoctoral fellow in the Richer lab recently started to work on the project on AR-regulated secreted factors and how they affect immune cells in the tumor microenvironment. She presented on this topic at the Endocrine Society meeting in June 2022 and will also present some of this work at the 9th Annual Metastatic Breast Cancer Research Conference, September 7-9, 2022.

Alyse Staley, M.S. began working under the mentorship of Dr. Richer, Clinical Oncologist Dr. Elias, and Biostatistician Dr. Gao on the 16-1001 and 16-1042 clinical trials while a graduate student at the University of Colorado School of Public Health. "I earned my MS in Biostatistics in May 2021 and continued to work on these projects after graduation, when I transitioned to my current position as a Research Instructor at the Cancer Center's Biostatistics Core. These projects served as one of my first exposures to clinical trials, cancer research, -omics analyses, and collaborating with researchers from other fields. Furthermore, this grant provided the

opportunity to be mentored by experienced researchers across disciplines. I especially appreciate my mentors' availability and patience in sharing their expertise and answering my questions."

How were the results disseminated to communities of interest? Dr. Richer gave the following lectures at national meetings:

April 2018 **AACR** Invited "Meet the Expert" session "Update on Potential for Targeting Androgen Receptors in Breast Cancer."

Aug 2018 **Endocrine Society of Australia** Two invited symposium lectures on the role of androgen receptors in breast cancer. Adelaide, AU

April 2019 **International Association of Breast Cancer Research**, Egmond aan Zee Netherlands, "Clinical biopsies and preclinical models reveal new therapeutic targets in ER mutant metastatic breast cancer"

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, Utah. "Targetable Pathways in metastatic ER+ BC resistant to aromatase inhibitor therapy."

Invited lectures:

Aug 2018 **The Westmead Institute for Medical Research, Sydney, AU "**Targeting AR in BC resistant to anti-estrogen therapy and TNBC"

Aug 2018 **University of Adelaide, Adelaide AU Cancer Biology and Reproductive Sciences** "Triple Negative Breast Cancer Hijacks a Trophoblast-Like Program of Immune Suppression"

*May 2020 **Northwestern University Feinberg School of Medicine Department of Pharmacology**- "New Targetable pathways in metastatic breast cancer." Rescheduled for September"

*Oct 2020 **The University of North Carolina Chapel Hill** Pathology Laboratory Medicine Molecular and Cellular Pathology "Steroid Hormone Milieu and female cancers: context is critical

*Oct 2020 **Reproductive & Developmental Biology Laboratory (RDBL), National Institute of Environmental Health Sciences NIEHS-** "Steroid Hormone Milieu and female cancers: context is critical."

Sept 2022 Speaker at **Royal College of Surgeons Institute Symposium in Dublin, Ireland** on Sex Steroids in Health and Disease, Sept 2nd.

Local lectures at the University of Colorado and in Denver

2019 Feb 22 Pathology Grand Rounds "Androgen Receptors in Breast Cancer-what have we learned?"

2019 April Endocrine Division Research Conference- "Estrogen Receptor Mutations in "Castrate Resistant Breast Cancer" – a role for androgen receptors?"

Feb 2019 Pathology Grand Rounds "Androgen Receptors in Breast Cancer-what have we learned?"

April 2019 Endocrine Division Research Conference- "Estrogen Receptor Mutations in "Castrate Resistant Breast Cancer" – a role for androgen receptors?"

Feb 2020 Advances in Breast Cancer: Updates from San Antonio and ESMO" conference CME for Colorado community caregivers Horizon CME

March 2022 Endocrine Division "Sex Steroid Hormone Receptor Action: Context is Critical"

We presented posters virtually on the trial for metastatic disease at SABCS 2020, AACR 2020 and SABCS 2021 and we have a submitted abstract for the neoadjuvant trial accepted to SABCS 2022 in the appendix.

- **What do you plan to do during the next reporting period to accomplish the goals?**

We will continue to analyze patient samples from the neoadjuvant 16-1042 trial clinicals. We just got the RPPA data and the gene expression data and are beginning to do the analyses. The metabolomics data will be completed by the end of August. The multiplex Polaris and Nanostring staining for immune cells are just starting for the 16-1042 trial. The last patient sample for the 16-1042 trial was obtained on 02/17/2022, the last PEPI score determined on March 25th and the RedCap data entry completed July 7th. We still have monitoring visits required to close out and lock the database (with the exception of the follow-up data still being collected) and those are scheduled to occur during August 2022. Only after the treatment phase data is validated can we unblind everyone as to which arm each of the patients were on and complete the analyses of the phosphoproteins and gene expression data and relate them to patient outcomes (PEPI score and changes resulting from treatment).

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**

These studies are helping to determine the role of androgen receptors in breast cancer and whether new anti-androgens might be utilized as therapy for breast cancers that fail to respond or reoccur while women are on current therapies such as anti-estrogens, trastuzumab or chemotherapy. These studies have provided preclinical evidence that the anti-androgen enzalutamide could serve as the first effective targeted therapy for a subset of triple negative breast cancers (TNBC). TNBC is the most aggressive type of breast cancer and there is currently no effective treatment for TNBCs with de novo or acquired resistance to chemotherapy. Our studies regarding timing (concurrent enzalutamide treatment with chemotherapy and enzalutamide versus sequential) provided valuable information for upcoming clinical trials- in fact, a trial like this is underway at MD Anderson for TNBC. Our studies on the *ESR1* mutations in metastatic ER+ breast cancer will also inform clinical trial design. Already it is evident that if the receptor does not mutate to become constitutively active in metastatic disease in the absence of ligand (most all patients were on aromatase inhibitors prior to going on this trial), the tumors have likely survived the estrogen deprivation by losing ER and PR expression and may no longer be candidates for effective aromatase inhibitor or fulvestrant or any ER targeting endocrine therapy. Those that did not get benefit from the combination of enzalutamide and fulvestrant in the 16-1001 trial had activation of the mTOR pathway detected by RPPA and or PIK3CA activating or PTEN inactivating mutations.

- **What was the impact on other disciplines?** Our studies of how steroid hormone receptors affect each other is pertinent to other cancers and development.
- **What was the impact on technology transfer?**
  - Transfer of results to entities in government or industry: The results of this project are also reported to our clinical industry partners Medivation Inc and Astellas Pharma who are running the clinical trials of enzalutamide in prostate and breast cancer. They are very interested in our preclinical results combining enzalutamide with other therapeutics currently being utilized in breast cancer since these results will guide the design of further industry or investigator initiated clinical trials. We filed a patent on the idea of looking at the AR to ER ratio in breast cancer and the company Ventana signed an agreement to pay the filing fees in Europe and to contract some additional sponsored research to design a clinical test to examine the ratio of these two receptors using their antibodies potentially simultaneously on the same section of tumor. We have approval from our institutional IRB, the SWOG Breast Cancer Translational Medicine and Executive Triage Committees, and the National Clinical Trials Network Core Correlative Sciences Committee (NCTN-CCSC) for Proposal #: **CSC0133** *"Effects of androgen receptor (AR) expression and activity on estrogen receptor alpha positive (ER+) breast cancer outcomes and immune cell status in the SWOG S0226 study."*

- **What was the impact on society beyond science and technology?**
- Since we have given reports of our research to several lay audiences in various community settings, we believe we are improving public knowledge regarding how hormones typically thought of as male hormones (such as androgens) are made by women and do affect women's health. Many women and men take testosterone for libido and transgender hormone therapy, so the long term effects on breast tissue, particularly in the absence of estrogen as in postmenopausal women, particularly those with breast cancer on aromatase inhibitor therapy.

5. **CHANGES/PROBLEMS:** Nothing to Report. We submitted one last NCE in late July to 02/14/2023 in order to complete the molecular characterization of the tissues and to pay the publication charges on the last two manuscripts on the two trials. We also requested to continue salary support for Drs. Richer and Elias to finish writing the paper on the 16-1042 trial, Nicole Spoelstra to finish IHC and multiplex, Tessa McSpadden to continue to manage the clinical trial follow up, Stephanie Hill to lock the clinical database at all 3 sites, and Tiffany Cull to continue regulatory management of the two protocols.

Changes in approach and reasons for change

- None
- **Actual or anticipated problems or delays and actions or plans to resolve them (see above regarding the NCE request).**
- **Changes that had a significant impact on expenditures**. Nothing to report.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.** No changes

  Significant changes in use or care of human subjects. None
- **Significant changes in use or care of vertebrate animals.** None
- **Significant changes in use of biohazards and/or select agents.** None

6. **PRODUCTS: We have new publications related to these grants listed below in red (for the ones this past year reporting period):**

Cochrane DR, Bernales S, Jacobsen BM., Cittelly DM, Howe EN, D'Amato NC, Spoelstra NS, Jean A, Jedlicka P, Torkko KC, Protter A, Elias AD and **JK Richer**. Role of the Androgen Receptor in Breast Cancer and Preclinical Analysis of Enzalutamide. BREAST CANCER RESEARCH 2014 Jan 22;16(1). PMID: 24451109 D'Amato NC, Jacobsen BM, Gordon MA, Babbs BL, Spoelstra NS, Carson Butterfield KT, Barton VN, Rogers TJ, Sartorius CA, Elias AD, Gertz, J and **JK Richer**. Cooperative Dynamics of AR and ER Activity in Breast Cancer. MOLECULAR CANCER RESEARCH 2016 Nov;14(11):1054-1067. PMID: 27565181

Barton VN, D'Amato NC, Gordon MA, Lind HT, Spoelstra NS, Babbs B, Heinz RE, Elias A, Jedlicka P, Jacobsen BM and **JK Richer**. Multiple molecular subtypes of triple negative breast cancer critically rely on androgen receptor and respond to Enzalutamide *in vivo*. MOL CANCER THER. 2015 Mar: 14(3):769-78 PMID: 25713333* Top most highly cited for this journal in 2015.

Christenson JL, Butterfield KT, Spoelstra NS, Norris JD, Josan JS, Pollock JA, McDonnell DP, Katzenellenbogen BS, Katzenellenbogen JA, and **JK Richer**. MMTV-PyMT and Derived Met-1 Mouse Mammary Tumor Cells as Models for Studying the Role of the Androgen Receptor in Triple-Negative Breast Cancer Progression. HORMONES AND CANCER. 2017 Apr;8(2):69-77.PMID: 28194662

Gordon MA, D'Amato NC , Gu H , Babbs B, Wulfkuhle JD , Petricoin EF, Gallagher RI, Dong T, Torkko KC, Liu B , Elias A and JK Richer. Synergy between androgen receptor antagonism and inhibition of mTOR

and HER2 in breast cancer. MOLECULAR CANCER THERAPEUTICS. 2017 Jul;16(7):1389-1400. PMID: 28468774

Barton VN., Christenson JL, Rogers TJ, Butterfield K, Babbs B, Spoelstra NS, D'Amato NC, Elias A, and JK Richer. Androgen receptor supports an anchorage independent, cancer stem cell like population in triple negative breast cancer. CANCER RESEARCH. 2017 Jul 1;77(13):3455-3466. PMID: 28512248

Williams MM, Spoelstra NS, Arnesen S, O'Neill KI, Christenson JL, Reese J, Torkko KC, Goodspeed A, Rosas E, Hanamura T, Sams SB, Li Z, Oesterreich S, Riggins RB, Jacobsen BM, Elias A, Gertz J, and **JK Richer.** Steroid hormone receptor and infiltrating immune cell status reveals therapeutic vulnerabilities of ESR1 mutant breast cancer. CANCER RES 2021 Feb 1;81(3):732-746 PMID: 33184106.

Rosas E, Roberts JT, O'Neill KI, Christenson JL, Williams MM, Hanamura T, Spoelstra NS, Vahrenkamp JF, Jason Gertz, **JK Richer** A Positive Feedback Loop Between TGFβ and Androgen Receptor Supports Triple-Negative Breast Cancer Anoikis Resistance. ENDOCRINOLOGY 2021 Feb 1;162(2) PMID: 3329492283.

Christenson JL, O'Neill KI, Williams MM, Nicole Spoelstra, Jones, KL, Trahan GD, Reese JM, Van Patten E, Elias A, Eisner JR, and **JK Richer** 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

Hanamura T, Christenson JL, O'Neill KI, Rosas E, Spoelstra NS, Williams MM, **Richer JK**. Secreted indicators of androgen receptor activity in breast cancer pre-clinical models. *Breast Cancer Res* 2021; 23(1):102. PMC8567567, PMID: 34736512

Oesterreich S, Li Z, Spoelstra NS, Sikora MJ, Sams SB, Elias A, **Richer JK**, and AV Lee. Mutual exclusivity of ESR1 and TP53 mutations in endocrine resistant metastatic breast cancer. *NPJ Breast Cancer*. 2022 May 10;8(1):62 PMID: 35538119

Elias, AD, Nicole S. Spoelstra, Alyse W. Staley, Sharon Sams, Lyndsey S. Crump, Gregory A. Vidal, Virginia F. Borges, Peter Kabo, Jennifer R. Diamond, Elena Shagisultanova, Anosheh Afghahi, Jose Mayordomo, Tessa McSpadden, Gloria Crawford, Angelo D'Alessandro, Kathryn L. Zolman, Adrie van Bokhoven , Yonghua Zhuang, Rosa I. Gallagher , Julia D. Wulfkuhle, Emanuel F. Petricoin III, Dexiang Gao, and **JK Richer**. Phase II trial of fulvestrant plus enzalutamide in ER+/HER2- advanced breast cancer. July 5 2022 *NPJ Breast Cancer* under review.

Elias, AD, Monica Fornier, Gregory A. Vidal, Sharon Sams, Nicole Spoelstra, Peter Kabos, Jennifer R. Diamond, Elena Shagisultanova, Lyndsey Crump, Rosa I Gallagher, Julia Wulfkuhle, Emanuel Petricoin, Kathryn Zolman, Stephanie Biller, Vida Alami, Alyse Staley, Tessa McSpadden, Virginia Borges, Dexiang Gao, Jennifer Richer. Randomized phase II trial of preoperative fulvestrant with or without enzalutamide for ER+/Her2- breast cancer. In preparation.

Reviews:

Barton VN, Gordon MA, Christenson J, D'Amato N, and **JK Richer.** Androgen receptor biology in triple negative breast cancer: A case for AR+ and quadruple negative disease subtypes. HORMONES AND CANCER 2015 Dec 6(5-6):206-13. PMID: 26201402

Barton VN, Gordon MA, **Richer JK**, Elias A. Anti-androgen therapy in triple-negative breast cancer. Ther Adv Med Oncol. 2016 Jul;8(4):305-8. PMID: 27482289

Gordon MA, Harrison B, **Richer JK**, Elias A. Anti-androgen therapy in breast cancer. *American Journal of Hematology/Oncology. 2016.*

Christenson JL, Trepel JB, Ali HY, Lee S, Eisner JR, Baskin-Bey ES, Elias AD, **Richer JK**. Harnessing a Different Dependency: How to Identify and Target Androgen Receptor-Positive Versus Quadruple-Negative Breast Cancer. HORMONES AND CANCER. 2018 Apr;9(2):82-94. doi: 10.1007/s12672-017-0314-5. Epub 2018 Jan 16. Review. PMID: 29340907.

- **Books or other non-periodical, one-time publications.** Nothing to report.
- **Other publications, conference papers, and presentations.**

Dr Richer and team gave the following lectures: with new this past year in red. See also "disseminated to communities of interest" above.

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."

Aug 2018 **Endocrine Society of Australia** Two invited symposium lectures on the role of androgen receptors in breast cancer. Adelaide, AU

Aug 2019 **Gordon Research Conference Hormones and Cancer**, Sunday River, Maine, "Hormone Deprivation Influences Breast Cancer Immune Suppression

Feb 2019 Pathology Grand Rounds "Androgen Receptors in Breast Cancer-what have we learned?"

Feb 2019 **MD Anderson - Symposium on Factors Impacting Immune Microenvironment –** "Carcinomas Hijack a Trophoblast-Like Program of Immune Suppression"

April 2019 Endocrine Division Research Conference- "Estrogen Receptor Mutations in "Castrate Resistant Breast Cancer" – a role for androgen receptors?"

April 2019 **International Association of Breast Cancer Research**, Egmond aan Zee Netherlands, "Clinical biopsies and preclinical models reveal therapeutic targets in ER mutant metastatic breast cancer"

Feb 2020 **Advances in Breast Cancer: Updates from San Antonio** and ESMO" conference CME for Colorado community caregivers Horizon CME

*May 2020 **Northwestern University Feinberg School of Medicine Department of Pharmacology**- "New Targetable pathways in metastatic breast cancer." Rescheduled for September"

*Oct 2020 **The University of North Carolina Chapel Hill** Pathology Laboratory Medicine Molecular and Cellular Pathology "Steroid Hormone Milieu and female cancers: context is critical

*Oct 2020 **Reproductive & Developmental Biology Laboratory (RDBL), National Institute of Environmental Health Sciences NIEHS-** "Steroid Hormone Milieu and female cancers: context is critical."

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"

May 2021* **Buenos Aires Breast Cancer Symposium**: From hormone receptors to the immune system. "Breast cancer hijacks a trophoblast-like program of immune suppression"

*remote due to Covid19

Sept 2022 **Royal College of Surgeons Institute Symposium in Dublin, Ireland** on Sex Steroids in Health and Disease, Sept 2nd.

Website(s) or other Internet site(s):

AACR/SABC video of 2017 December meeting lecture in the educational section by Dr. Jennifer Richer- video

Technologies or techniques. We may file a patent on a multiplex panel that we developed to stain for ER, PR, AR and GR the same tumor section. We won an award for these images: Endocrine Images Award Winners | Endocrine Society

- **Inventions, patent applications, and/or licenses**

  Richer *et. al*., PCT Patent Application WO 2014/031164 filed March 15, 2013, "Methods for Determining Breast Cancer Treatment." U.S. Patent Application No. 14/423,133, filed February 22, 2015Issued in European Countries European Patent 28888594, published August 15, 2018 and in the US allowed by the United States Patent and Trademark Office on August 27, 2018.

  Protter and JK Richer, PCT Patent Application PCT/US2012/48471 Serial No. 14/236,036 filed on January 29, 2014 "Treatment of Breast Cancer."

  See above on techniques as well.

- **Other Products**

  data or databases- we now have databases of genes expression data from the following experiments.

  ER+ MCF7 breast cancer cells treated in vitro with vehicle, enzalutamide alone, estradiol alone (E2), E2 plus enzalutamide for 48 hrs.

  ER+ MCF7 breast cancer cells grown as xenografts in nude mice treated with E2, E2 plus tamoxifen, or E2 plus enzalutamide**.**

  HCC1806 TNBC breast cancer line treated in vitro with either vehicle, DHT, enzalutamide alone, DHT plus enzalutamide.

  SUM159 treated in vivo.

  MDA-453 treated with CDK4/6 inhibitor

  TNBC PDX HCI-009 treated in vivo with or without DHT and gene changes list from RNAseq

- **biospecimen collections**;

  Formalin fixed paraffin embedded xenograft tumors from the following experiments:

  MCF7 tumors grown in nude mice and treated with either E2, E2 plus tamoxifen, E2 plus enzalutamide or in a separate experment, the same treatments plus the combination of E2 plus enzalutamide and tamoxifen.

  Triple negative breast cancer (TNBC) cell line SUM159PT grown as xenograft tumors in mice treated with control rodent chow or enzalutamide containing chow.

  TNBC cell line HCC1806 grown as xenograft tumors in mice treated with control rodent chow or enzalutamide containing chow.

  TNBC PDX-009 tumors grown in mice with or without DHT and gene expression change RNA-seq data

- research material (e.g., Germplasm; cell lines, DNA probes, animal models); We have generated luciferase labelled breast cancer cell lines to image by IVIS and put nuclear red and green expression vectors in these lines to utilize the Incucyte machine to count the number of red or green nuclei to do real time proliferation assays with enzalutamide alone or in combination with standard therapies for breast cancer.
- Biopsies (frozen and FFPE) cores obtained from the clinical trial are stored with our Pathology Biobank.
- Biospyder gene expression data from serial biopsies from both clinical trials
- RPPA data on phosphor-protein expression from specimens of metastatic and primary tumor serial biopsies from both trials.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Drs. Elias and Richer and her technician and bioinformatics staff Andrew Goodspeed and Alyse Stayley to continue to analyze the molecular correlates from the study including gene expression, RPPA and immunohistochemstry in a blinded fashion. Technician Nicole Spoelstra does the immunostaining of biopsies from the clinical aims and pathologist Sharon Sams scores them. They are all paid for now from Dr. Elias's partnering grant. We also requested continued salary alary support for Tessa McSpadden to continue to manage the clinical trial follow up, Stephanie Hill to lock the clinical database at all 3 sites, and Tiffany Cull to continue regulatory management of the two protocols.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** No changes in active support for the PD/PI(s) or senior/key personnel.

- **What other organizations were involved as partners?** See above regarding other sites where the clinical trials were open.

SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** Partnering PI, Dr. Anthony Elias has sent a separate report with the same details on the clinical trial progress.
- **APPENDICES:**

  Manuscript summarizing the findings from the trial of fulvestrant plus enzalutamide in metastatic ER+ Breast cancer (16-1001 trial).

  Abstract sent to SABCS for meeting in Dec 2022 in appendix.

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

Authors: Anthony D. Elias[1], Nicole S. Spoelstra[2], Alyse W. Staley[3], Sharon Sams[2], Lyndsey S. Crump[2], Gregory A. Vidal[4], Virginia F. Borges[1], Peter Kabos[1], Jennifer R. Diamond[1], Elena Shagisultanova[1], Anosheh Afghahi[1], Jose Mayordomo[1], Tessa McSpadden[5], Gloria Crawford[6], Angelo D'Alessandro[7], Kathryn L. Zolman[2], Adrie van Bokhoven[2], Yonghua Zhuang[3], Rosa I. Gallagher[8], Julia D. Wulfkuhle[8], Emanuel F. Petricoin III[8], Dexiang Gao[3], and Jennifer K. Richer[2,*]

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Running title: Fulvestrant plus enzalutamide in advanced breast cancer

Keywords: enzalutamide, anti-androgen, metastatic breast cancer, endocrine resistance

ADDITIONAL INFORMATION

Funding: Department of Defense Breast Cancer Research Program Clinical Translational Award BC120183 W81XWH-13-1-0090/91

Conflicts of Interest Statement: The authors have no relevant COI.

Word count: 5187

Total number of figures and tables: 6

Table 1: Descriptive 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			
N	33	26	59
>0	25 (75.8%)	24 (92.3%)	49 (83.1%)
0	8 (24.2%)	2 (7.7%)	10 (16.9%)
PFS (Months)***			
N	33	26	59
Median (Range)	3.7 (0.9, 15.2)	3.6 (2.5, 5.8)	3.7 (0.9, 15.2)

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

**"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.

***Progression free survival (PFS) 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.

**"Yes" indicates patients who had surgery during the study and "No" indicates patients who did not have surgery during the study. Three patients did not have surgery: one insurance related, and two physician decision.
***PFS 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.

Table 2: Summary of Treatment-Related Adverse Events (trAEs)

	Combination Arm (N=33)	Fulvestrant Arm (N=26)	Total (N=59)
Patients with any trAE(s)	28 (84.8%)	20 (77%)	48 (81.4%)
Patients with any Grade 3/4 trAE(s)	1 (3%)	2 (7.7%)	3 (5.1%)
Grade 1/2 trAEs ≥10%*			
Fatigue	16 (48.5%)	7 (26.9%)	23 (39%)
Hot Flashes	14 (42.4%)	8 (30.8%)	22 (37.3%)
Nausea	12 (36.4%)	2 (7.7%)	14 (23.7%)
Headache	11 (33.3%)	2 (7.7%)	13 (22%)
Arthralgia	7 (21.2%)	2 (7.7%)	9 (15.3%)
Diarrhea	6 (18.2%)	1 (3.8%)	7 (11.9%)
Insomnia	7 (21.2%)	0	7 (11.9%)
Cognitive Disturbance	4 (12.1%)	1 (3.8%)	5 (8.5%)
Constipation	2 (6.1%)	3 (11.5%)	5 (8.5%)
Dizziness	4 (12.1%)	0	4 (6.8%)

Table includes trAEs determined to be probably or possibly related to treatment.
* Occurred in ≥10% of patients in total or in at least one arm.

Conclusions: The combination of fulvestrant plus enzalutamide had manageable side effects. PEPI score of 0 was achieved more frequently on the combination arm although this difference did not quite meet pre-specified statistical significance within the statistical power of the sample size ($p = 0.16$). Extensive molecular studies of paired fresh biopsies from pretreatment and at 4 weeks are underway to evaluate predictive biomarkers. These analyses and correlations with clinical outcome will be described.

ABSTRACT

Purpose: This clinical trial combined fulvestrant with the anti-androgen enzalutamide in women with metastatic ER+/HER2- breast cancer.

Experimental Design: Eligible patients were women with ECOG 0-2, ER+/HER2- measurable or evaluable metastatic breast cancer. Prior fulvestrant was allowed. Fulvestrant was administered at 500 mg IM 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 clinical benefit rate at 24 weeks (CBR24).

Results: Median age was 61 years (46-87); PS 1 (0-1); a 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. *S*ignificant ($p<0.1$) univariate relationships existed between PFS and the following variables: ER and AR percent positivity, 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.

Conclusions: Fulvestrant plus enzalutamide had manageable side effects and the primary endpoint of CBR24 was 25% in this population of women with persistent metastatic ER+/HER2- breast cancer. 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, this combination warrants further investigation in the treatment of ER+ metastatic disease.

TRANSLATIONAL RELEVANCE

Although a high ratio of AR to ER protein in primary breast cancer is associated with resistance to ER-targeting therapies, it is unclear whether AR might serve as a therapeutic target in heavily pretreated advanced ER+ breast cancer. We therefore sought to test whether AR inhibition is clinically efficacious in this setting. Enzalutamide is a potent inhibitor of AR approved for prostate cancer treatment. In this phase II study, women with heavily pretreated metastatic ER+/HER2- breast cancer received the combination of fulvestrant plus enzalutamide, with tolerable side effects. Approximately 25% of patients experienced a clinical benefit of greater than 24 weeks on therapy, including 2 whose disease was progressing on prior fulvestrant, suggesting benefit from the anti-androgen. Serial biopsies were correlated with clinical outcomes to gain insight into characteristics of response to fulvestrant plus enzalutamide treatment.

INTRODUCTION

Breast cancer (BC) is a genetically heterogeneous and biologically diverse disease. We currently subdivide breast cancer 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+ disease (1). 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+ BC (2). The predominately nuclear localization of AR by IHC (2) 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 decreases (3).

The functional role of AR in ER+ BC remains controversial (4), with confusion arising from the fact that when estradiol is present, androgens decrease ER-mediated proliferation in cell line and xenograft models (5). 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 epithelium (10). 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 alone (9). 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 models (5). 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 significantly greater benefit of enzalutamide (20). These complexities emphasize the importance of clinical context and hormonal milieu when considering AR action in BC (21). Clearly, AR does influence BC biology, and high AR relative 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 benefit.

The use of the anti-androgen enzalutamide showed efficacy in women with TNBC (22), 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 men (23). 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 growth (3). 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. Secondary objectives were to confirm the safety profile of the combination, response rate, and progression-free survival at 24 weeks. Serial biopsies were obtained pretreatment and at end of the fourth week of treatment (hence referred to as week 5) to evaluate the effects of treatment on tumor and relationship to clinical outcomes.

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 day 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. The study was conducted at the Universities of Colorado and Tennessee. The study protocol and its amendments were approved by the respective Institutional Review Boards. All patients provided written informed consent prior to participating in the study. The study was conducted under the principles of the World Medical Association, Declaration of Helsinki, and Good Clinical Practice guidelines of the International Conference on Harmonisation. 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 breast cancer determined to be ER positive and Her2 negative. Prior anti-androgens treatment was not allowed. Prior fulvestrant was allowed if the treating physician felt that retreatment with fulvestrant was clinically indicated. 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 ~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 adverse events (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 Adverse Events (CTCAE), version 4.03. SAEs were also evaluated by Astellas Pharma Global Development – United States. A monthly teleconference was held amongst the institutional investigators to review patients and adverse events. An institutional Data Safety and Monitoring Committee at 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 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 were obtained if available. A tumor biopsy at time of progression was requested, but optional. Lithium-heparin (LiHep) plasma samples were obtained at the same intervals. After collection LiHep vacutainers were centrifuged for 20 minutes (600 x g), separated plasma was transferred to a 15mL conical tube, centrifuged a second time for 15 minutes (1500 x g), aliquoted into 500uL aliquots, and stored at -80ºC until analysis.

Statistical analysis

Sample size

CBR24 was used as the primary endpoint for sample size. Assuming the undesirable rate of 10% and desirable 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.

Data Analysis Considerations

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 progression free survival

The clinical benefit rate was defined as the percent of patients with 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; ≥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 central nervous system disease enrolled in clinical trial NCT02953860. Formalin-fixed paraffin-embedded (FFPE) sections were analyzed for mutations in *ESR1* exon 8 and 67 other gene hotspots om 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 node (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, PR, AR, and GR as well as Ki67 and cleaved caspase 3 as described previously (6). To assess the association between each IHC variable with time, univariate linear mixed models (LMMs) were run separately for each variable with time as the predictor of interest. Log transformations of each outcome were considered based on visual model diagnostics. A random intercept was included to account for the variability across patients.

Reverse-Phase Protein Microarray (RPPA)

Enriched epithelial cell subpopulations were isolated from 8um cryosections (> 95% purity) using an Arcturus Pixcell IIe Laser Capture Microdissection system (Arcturus, Mountain View, CA, USA) as described (26). 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 2x SDS-PAGE Sample Buffer (ThermoFisher) plus 2.5% beta-mercaptoethanol (BME) at a concentration of approximately 500-600 cells per 1 uL Samples were heated at 100C for 5min, briefly centrifuged and stored at -20C until printed. Lysates were printed in triplicate spots (approx. 10nL 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 purposes (27). Antibodies used on the arrays were validated before use (28). Immunostaining was performed as previously described (29). Each slide was probed with one primary antibody targeting each of 158 proteins of interest **(Supplemental Table 1)**. Biotinylated goat anti-rabbit (1:7,500, 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, Mannedorf, Switzerland) and analyzed with MicroVigene software Version 5.6. (Vigenetech, Carlisle, MA, USA). 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 $\log_2$-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 phospho-protein, AR, and ER were then assessed. Each $\log_2$ 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 adjusted for post-treatment measurements. The association between the fold change of each protein and the fold change of AR or ER were similarly assessed with the change in AR or ER as the outcome and 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 package (30) 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 package (31). 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 described (32). Briefly, plasma (20 microliters) was extracted in 980 µl 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 18,000g 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 minute gradient as described (32-34). 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 (32-34).

Baseline and week 5 Metabolomics data analysis was performed via MetaboAnalyst 5.0 (35) by comparing autoscale normalized data for "Short PFS" and "Long PFS" groups at baseline and after treatment. In addition, Log_2-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.

Data Availability

The data generated in this study are available upon request from the corresponding author.

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 [0-1]. 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. Fatigue, nausea, and achiness were most common. Cognitive dysfunction described as “difficulty concentrating" was reported in 5 patients and resolved upon completion of treatment. Adverse events greater than 20 percent 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 adverse events 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 to 44.9) participants had stable disease and no participants had partial response **(Figure 1A).** The median time to progression was 8 weeks (95% CI: 8 to 20) **(Figure 1B)**.

Demographic and clinical risk factors of progression

Univariate survival analysis

*S*ignificant (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 ≥ 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 to 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 indicated by color **(Figure 1E).** No other factors of interest, including prior exposure to fulvestrant or everolimus, had significant univariate associations with PFS **(Table 3, Figure 1E).**

Multivariate Cox Proportional Hazard survival analysis

Dichotomous AR and ER percent positive 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 to 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 to 14.35) times the hazard for participants with ER ≥ 10 % positive, after controlling for AR percent positive (p=0.007) **(Table 3, Figure 1D)**.

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 to 101.6; p=0.003) units lower at week 5 than at baseline and Ki67 was 10.9 (95% CI 0.1 to 21.8; p=0.05) percentage points lower at week 5 than at baseline. There was not enough evidence to conclude that any other IHC variable had a significant difference between baseline and week 5 of treatment **(Figure 1C)**. 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 **(Figure 1D)**.

Reverse Phase Protein Array demonstrates activation of the mTOR pathway in tumors from patients in the short PFS group

Phosphorylated proteins in the mTOR pathway had higher baseline expression levels in the "Short PFS" defined in all RPPA analyses as PFS ≤ 60 days compared to those who experienced "Long PFS" defined as PFS>24 weeks. 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 p7056K T389 (p=0.043) were significantly higher in the Short PFS group compared to the Long PFS group **(Figure 2A&B).** Similar results were obtained when Short PFS was defined as <24 weeks. This suggests mTOR activation in the pre-treatment specimens of patients with a short time to progression **(Figure 2C).** However, the differences in the baseline abundance of each mTOR protein were not significantly different between 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 **(Figure 2D&E).**

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 **(Supplemental Table 2a**, 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 **(Supplemental Table 2b).** 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 **(Supplemental Tables 3a&3b)**. 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 **(Supplemental Table 4a&4b)**. Response to estradiol and epithelial cell proliferation corresponded with baseline ER levels and the change in ER with treatment **(Supplemental Table 4b)**. 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 **(Supplemental Table 5a)** and some of the same pathways and embryonic development changed with AR with treatment as well **Supplemental Table 5b.**

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 (19,36,37), 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 lower level of amino acids (L-arginine, citrulline, glutamate, lysine, methionine) and purine breakdown and deamination products (inosine, xanthine, guanosine) at baseline (**Figure 3A**). Following treatment, purine oxidation products (hypoxanthine, xanthine, 5-hydroxyiusourate) and amino acids (asparagine, glutamine, glutamate) were higher in the Short PFS group (**Figure 3B**). However, serine, citrulline and the main soluble intracellular antioxidant tripeptideglutathione, were higher in plasma from Long PFS patients (**Figure 3B**). This is relevant considering the role of mTOR in amino acid sensing – especially serine (38). An increased consumption of fatty acids and preservation of plasma acyl-carnitine levels was 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.

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 (39, 40) 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 absent (3). 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 depletion (41). 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 function (42), 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 (19,43). *PIK3CA* and *PTEN* mutations are frequent in metastatic ER+ breast cancer (44,45). 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 completed (20). Overall, 247 patients with ER+ BC were randomized into two cohorts (one with no prior endocrine therapy for metastatic disease and 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 reduced risk

of progression or death compared to control arm [HR, 0.24 (95% CI, 0.10–0.60); P=0.0011, 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 introduction (46). Interestingly, enobosarm, a selective androgen receptor modulator (SARM) with both agonist and antagonist activity depending on tissue type, showed activity in preclinical (5,47) and clinical studies (48). In women with endocrine sensitive ER+ BC on adjuvant ET for at least 3 years or who responded to 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) (48). 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% CBR24 (49).

Strengths and Limitations

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. Sample size is limited; thus, all correlations are by nature exploratory. Future research must be conducted to evaluate the reproducibility of the results. Regarding 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 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 (50,51). 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.

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678 **Table 1: Patient Characteristics**

Consented	38 (6 screen fail)
Eligible & Evaluable	32 (100.0%)
Age at Time of Consent (Years) Median (Range)	61 (46, 87)
ECOG PS Median (Range)	1 (0, 1)
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)
≥ 3 Metastatic Sites	17 (53.1%)
Bone	24 (75.0%)
Bone Only	2 (6.2%)
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**	18 (56.2%)
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.

[a]Enza dose in stage 2 was 160 mg/day.

[b]Two patients had ECOG PS of 2.

[d]Patient with initial diagnosis of triple-negative breast cancer, but was ER+/PgR+ in metastatic setting.

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Table 2: Adverse Events

AE	Any Grade (N=32)	G3 Related (N=32)
Fatigue	17 (53.1%)	2 (6.2%)
Hot Flashes	6 (18.8%)	0
Insomnia	6 (18.8%)	0
Anxiety	4 (12.5%)	0
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
Cognitive Disorder	5 (15.6%)	0
Lightheaded	6 (18.8%)	0
Headache	11 (34.4%)	0
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.

Table 3: Univariate and Multivariate Analyses

Time to Disease Progression

Univariate Predictors	*Hazard Ratio*	*CI*	*p*
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 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)	1.10	0.52 – 2.34	0.797
PTEN and/or *PIK3CA* (N=29)	2.02	0.89 – 4.56	**0.092**
Multivariate Predictors (N=27)	*Hazard Ratio*	*CI*	*p*
AR<10% Positive	2.46	0.97 – 6.22	0.057
ER <10% Positive	4.69	1.53 –14.35	**0.007**

* “Ki67 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. 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. The hazard of disease progression for participants with AR<10% positive was 2.28 (95% CI: 0.93 to 5.62; p=0.073) times the hazard for participants with AR≥10 % positive and the hazard of disease progression for participants with ER<10% positive was 4.32 (95% CI: 1.46 to 12.83) times the hazard for participants with ER ≥ 10 % positive (p=0.008). 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≥10% positive. The hazard of disease progression for patients with PTEN and/or PIK3CA was 1.99 (95% CI: 0.88 to 4.50; p=0.096) times the hazard for participants with neither PTEN and/or PIK3CA.

All other univariate associations of interest were non-significant predictors of progression, with p-values greater than 0.2. The hazard of disease progression for participants with AR<10% positive was 2.46 (95% CI: 0.97 to 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 to 14.35) times the hazard for participants with ER ≥ 10 % positive, after controlling for AR percent positive (p=0.007).

A

Clinical Benefit Rate*	Eligible for Efficacy (N)***	Stable Disease (N)	Partial Response (N)	CBR (95% CI)
CBR12**	28	9	1	35.7% (18.6%, 55.9%)
CBR24**	28	7	0	25.0% (10.7%-44.9%)

CBR, clinical benefit rate defined as complete response, partial response, or stable disease; CBR 12, CBR at 12 weeks; CBR 24, CBR at 24 weeks; CI, confidence interval; Enza, enzalutamide; RECIST, Response Evaluation Criteria in Solid Tumors version 1.1.

*The assessment of the target lesion and the non-target lesion were combined

***28 patients had evaluable disease, had taken Enza for at least 4 weeks, and had at least one post-baseline tumor assessment

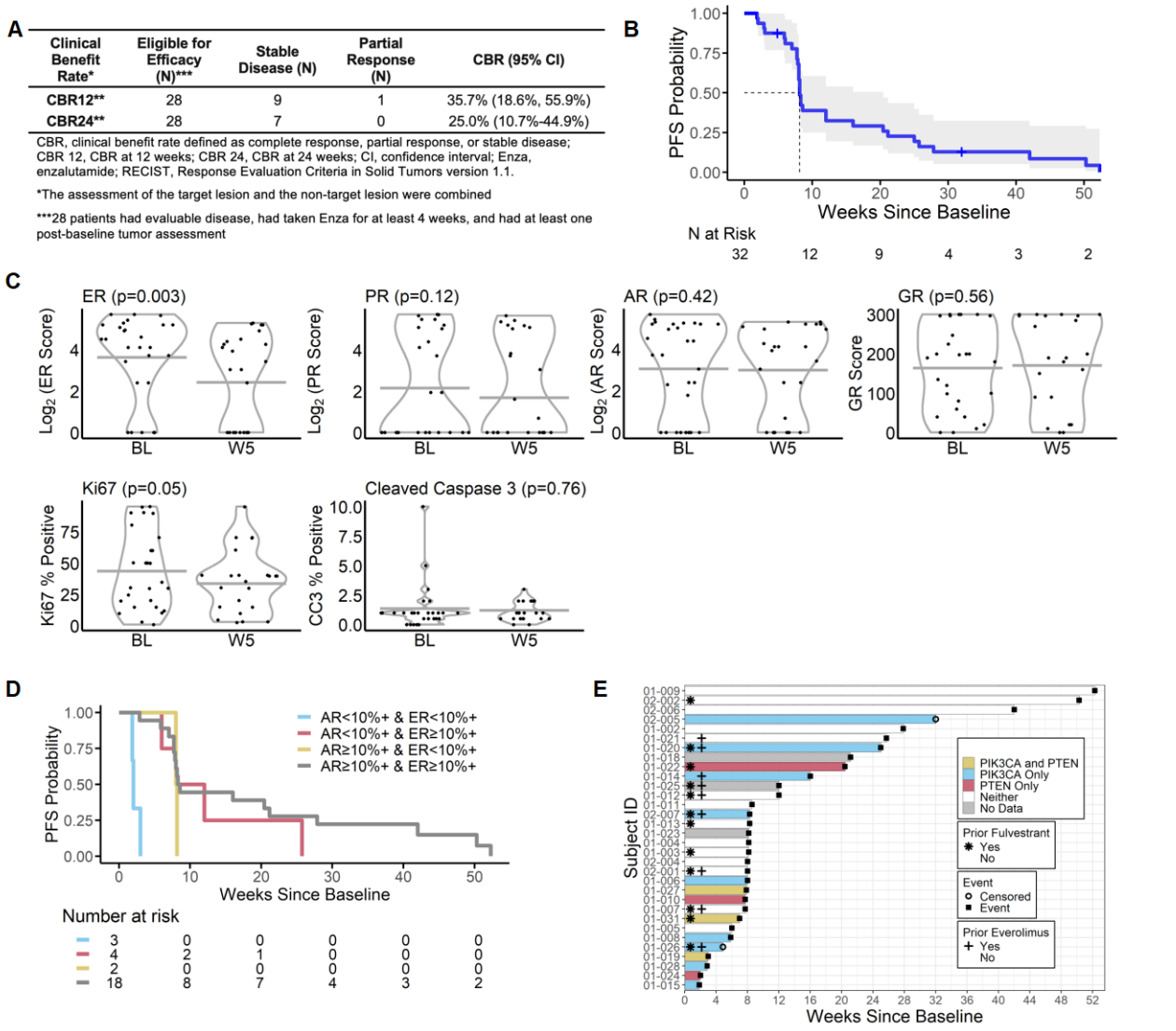

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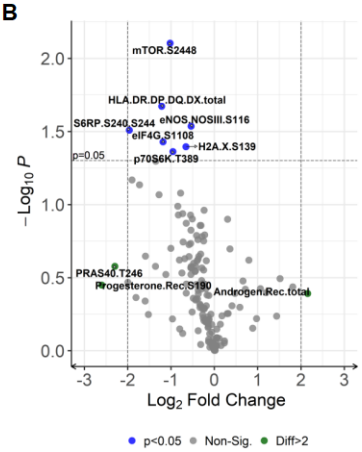
Figure 1. Efficacy of enzalutamide plus fulvestrant in advanced ER+/HER2- breast cancer patients. A) Clinical benefit rate at 12 weeks (CBR12) and 24 weeks (CBR24). **B)** Kaplan-Meier of progression free survival (PFS) in all patients treated with enzalutamide plus fulvestrant. Censored times are marked with vertical dashes and 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. Grey horizontal lines indicate LMM-predicted averages with corresponding p-values and black points indicate observed values. **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, prior fulvestrant is presented with a star, and prior everolimus is presented with a plus.

A

Protein	Long PFS Mean (SD)[b]	Short PFS Mean (SD)[b]	Mean Diff[a]	t-test p-value[c]
mTOR.S2448	14.27 (0.91)	15.29 (0.64)	-1.02	**0.008**
HLA.DR.DP.DQ.DX.total	11.54 (0.14)	12.76 (1.29)	-1.22	**0.021**
eNOS.NOSIII.S116	14.17 (0.33)	14.71 (0.52)	-0.54	**0.029**
S6RP.S240.S244	10.57 (1.45)	12.53 (2.04)	-1.96	**0.031**
eIF4G.S1108	13.55 (1.32)	14.74 (1.07)	-1.19	**0.037**
H2A.X.S139	14.24 (0.69)	14.9 (0.6)	-0.66	**0.04**
p70S6K.T389	13.71 (0.98)	14.66 (0.95)	-0.95	**0.043**
MET.Y1234.Y1235	11.51 (1.24)	12.86 (1.51)	-1.35	0.05
PRAS40.T246	10.99 (5.03)	13.29 (4.11)	-2.30	0.264

[a]Difference is defined as the mean baseline expression amongst patients in the "Long PFS" group minus the mean baseline expression amongst patients in the "Short PFS" group. A positive difference indicates that the Long PFS group had a larger mean baseline expression than the Short PFS group. A negative difference indicates that the Short PFS group had a larger mean baseline expression than the Long PFS group. The corresponding p-values indicate whether this difference in baseline expression was statistically significant. [b]Means and SDs are reported in $\log_2$ units. Medians, minimums, and maximums are reported in original units. [c]P-value from $\log_2$ moderated t-test.

B



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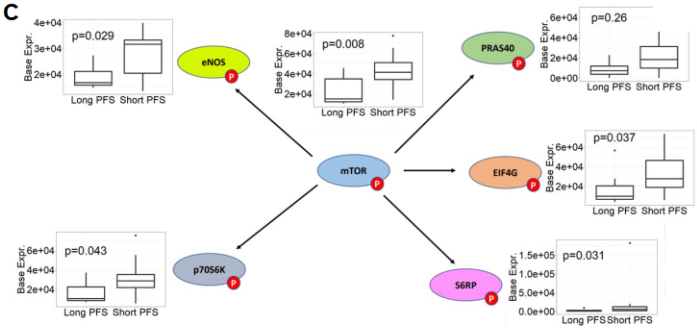

D

Protein	Long PFS Mean Change (SD)[1]	Short PFS Mean Change (SD)[1]	LogFC	t-test p-value[2]
cABL.Y245	1.57 (3.9)	-3.1 (4.69)	4.68	**0.03**
LKB1.S334	0.35 (0.3)	-0.03 (0.28)	0.38	**0.037**
PAK1.S199.S204.PAK2.S192.S197	0.42 (0.59)	-0.56 (1.08)	0.98	**0.037**
S6RP.S240.S244	1.48 (1.69)	-0.21 (1.72)	1.69	**0.042**
eIF4G.S1108	0.76 (1.26)	-0.29 (1.3)	1.05	0.091
EGFR.total	0.35 (0.53)	-0.12 (0.69)	0.47	0.141
RB.S780	-1.25 (5.06)	2.31 (5.21)	-3.56	0.141
S6RP.S235.S236	1.21 (1.83)	-0.04 (1.86)	1.25	0.152
cKIT.Y719	0.27 (0.29)	-0.08 (0.59)	0.35	0.182
TROP2.total	-0.72 (2.1)	0.23 (1.16)	-0.95	0.19
ATP.Citrate.Lyase.S454	0.34 (0.56)	-0.02 (0.53)	0.36	0.193
mTOR.S2448	0.41 (1.14)	-0.14 (0.71)	0.55	0.195
STAT1.Y701	0.37 (0.68)	-0.39 (1.42)	0.75	0.196

[1]Change is defined as the difference between baseline and week 5 of treatment; a positive change indicates a greater expression at week 5 than at baseline and a negative change indicates a greater expression at baseline than at week 5. All values are in $\log_2$ units.[2]P-value from $\log_2$ moderated t-test.

E

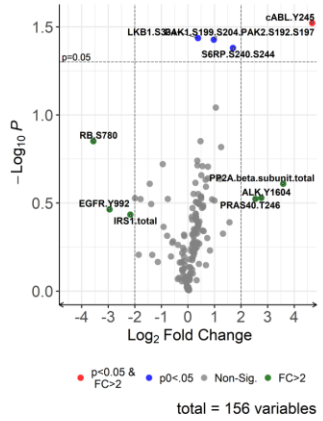

Figure 2. RPPA analysis of frozen core biopsies from tumors at baseline and following enzalutamide plus fulvestrant. A) The phospho-proteins from pretreatment tissue (at baseline) that had significantly ($p<0.05$) different expression across the Long versus Short PFS groups according to the log2 moderated t-test. **B)** Volcano plot of differences in baseline detection across the Long and Short PFS groups. **C)** Differentially expressed phospho-proteins in the mTOR signaling pathway in Long PFS versus Short PFS at baseline. **D)** Differentially ($p<0.2$) detected proteins between baseline and week 5 of treatment in the Short PFS versus Long PFS groups according to the log2 moderated t-test. **E)** Volcano plot of differences in fold change detection across Long and Short PFS groups.

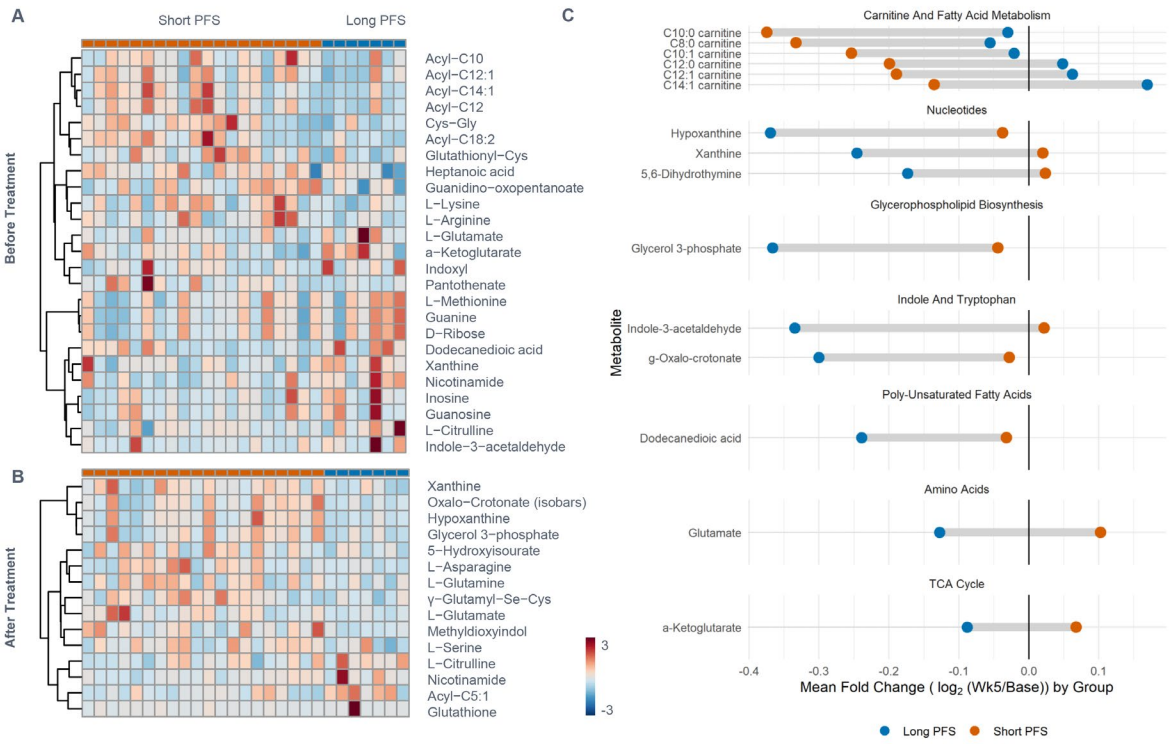

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Figure 3. Analysis of plasma metabolomics performed via UHPLC-MS at baseline and after treatment. Metabolites with significant differences between Long and Short PFS are presented in heatmaps **A)** at baseline and **B)** after treatment. **C)** The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

16-1042 SABCS 2022 abstract: Randomized phase II trial of preoperative fulvestrant with or without enzalutamide for ER+/Her2- breast cancer

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Background: Almost all ER+ breast cancers (BC) express androgen receptor (AR), but its function is uncertain. AR expression is associated with more indolent tumors, however high AR expression relative to ER is associated with endocrine resistance. In the absence of estradiol or if ER function is blocked, preclinical data would suggest that AR can take over to signal cell survival and proliferation. This non-blinded randomized phase II trial of neoadjuvant fulvestrant with or without enzalutamide was performed in women with T2 or greater ER+/Her2- primary BC to determine if the addition of AR blockade would increase the percentage of patients with limited residual tumor at time of surgery as measured by modified preoperative endocrine predictive index (PEPI) score.

Methods: Details of the methods were included in a SABCS abstract from 2020 (Elias et al, PS12-06). Eligible patients were women with ECOG 0-2, ER+/Her2- primary breast cancer cT2 or greater. A total of 4 months of therapy was given (fulvestrant 500 mg IM weeks 1, 3, 5, 9, and 13). Patients were randomized to receive enzalutamide 160 mg po daily on a continual basis for 16 weeks. Stratification factors were clinical node status (N0 vs N1/2) and T-stage (T2 vs T3/4). Surgery was planned for week 17. Fresh tumor biopsies were required at study entry and at ~4 weeks on therapy. Tissue, both fresh frozen and FFPE, was also obtained at time of surgery. The modified PEPI score at time of surgery was the primary endpoint for efficacy. The minimax two-stage design had 80% power with the type I error rate of 0.08.

Results: The trial has completed accrual and all patients have completed protocol-mandated therapy. 69 patients were consented of whom 59 were treated. Table 1 describes the patient population and surgical results. Table 2 describes toxicities felt to be possibly or probably related to treatment.