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

PRINCIPAL INVESTIGATOR: Dr. Jennifer Richer

CONTRACTING ORGANIZATION: University of Colorado

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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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Award Number W81XWH-13-1-0090 Annual Report (Year 8)

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

Initiating PI: Jennifer Richer, PhD

Collaborating/Partnering PI: Anthony D Elias, MD

Contracting Organization: University of Colorado Anschutz Medical Campus

Report Date: 8/15/2020-8/14/2021 (in no cost extension)

Type of Report: Eighth Annual Progress Report

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 assays on biopsies from the clinical trials, which are nearing completion of enrollment.

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 is to rapidly generate preclinical data in the laboratory of Jennifer Richer, Ph.D., testing Enza 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. Currently we are preparing four manuscripts on AR in breast cancer that focus on genes regulated by AR in AR+ TNBC PDX and how AR positive TNBC may be targeted with CDK4/6 inhibitors. We also are writing up a study of AR expression in over 1000 cases of invasive breast cancer and how it affects clinical outcome. The latest 3 papers were published this year. Finally as the last part of this grant in the no cost extension year as we finish the clinical trials, we will write up the results of the molecular analyses of gene mutations, expression levels and phospho proteins are altered in ER+ breast cancers treated either in the neoajuvant or metastatic setting with fulvestrant versus fulvestrant plus Enza.

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?

TASK1- Evaluate enzalutamide in combination with anti-estrogen therapy in ER+/AR+ BC lines (MCF7, BCK4) and a ER+/AR+ patient derived xenograft. Months 1-4. 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. *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. Since Fulvestrant must be given IM in oil, being able to reduce the effective dose necessary would be clinically useful.

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. 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 4. Examine enzalutamide in combination with an mTOR inhibitor (Afinitor/everolimus). (100% complete) published in (Gordon MA *et al* MOL CA THER 2017). See directly above.

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*. (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.

Conclusions 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 in TNBC with very few cells positive for AR, perhaps by targeting a different cell population.

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.
- **This year (2020/2021) we published two additional papersChristenson 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**. 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. In this paper 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, perhaps 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 3 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.

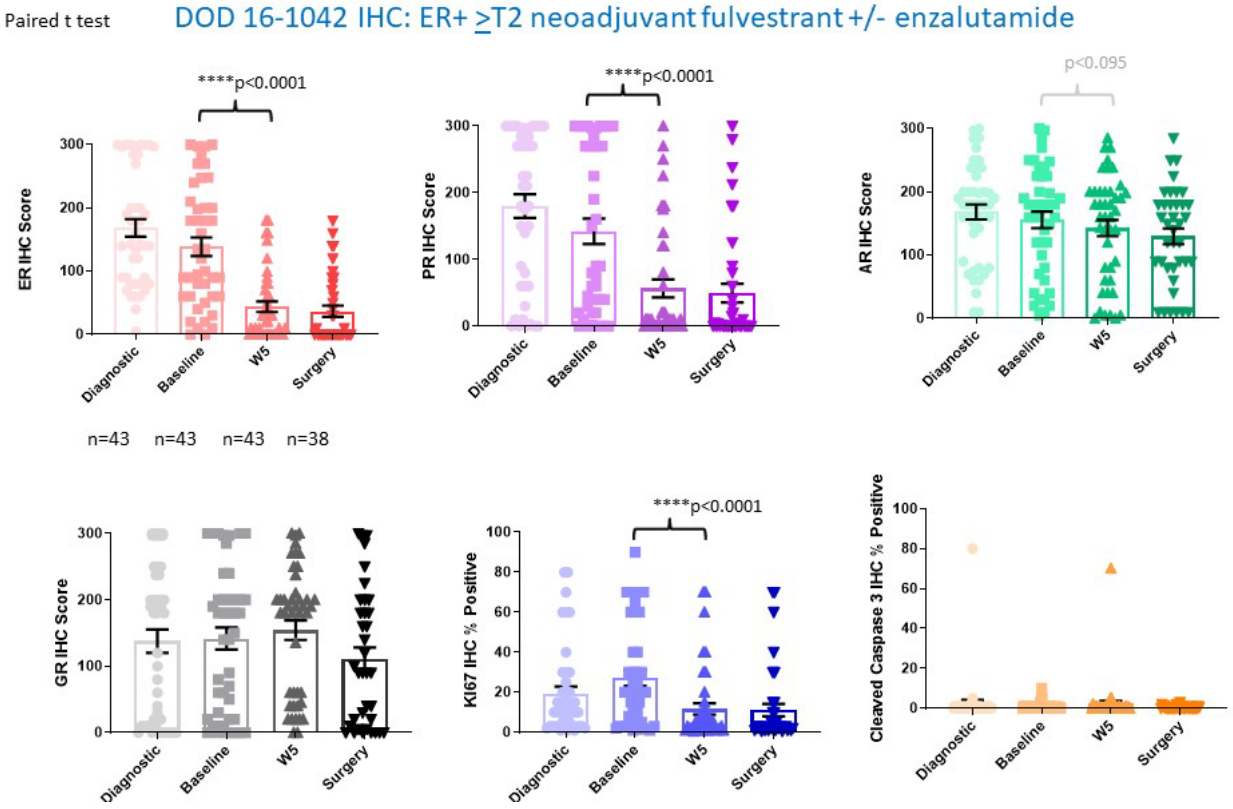
Summary for this aim:

- 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.
- However, since the last annual report, we did find and 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**.** A Positive Androgen receptor (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**

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. The trial for metastatic breast cancer 16-1001 just completed and we are writing a manuscript on the results of that trial. We presented posters at the San Antonio BC Conference and AACR virtually last year (see below in outcomes section).

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. Currently 68 patients consented. 61 enrolled (3 screen failures and 4 withdrawals prior to enrollment), and 60 randomized and treated (1 patient withdrew prior to

treatment). We are analyzing the immunohistochemistry for ER, PR, AR and GR as well as Ki67 and cleaved caspase 3 for proliferation and apoptosis. All IHC was scored by breast cancer pathologist Sharon Sams, MD. See results (Figure 1 below).



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. We are presenting the results in posters at SABCS and AACR. We published a paper with some of the pretreatment data in early 2021 Steroid Hormone Receptor and Infiltrating Immune Cell Status Reveals Therapeutic Vulnerabilities of *ESR1*-Mutant Breast Cancer.

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, **Richer JK.** Cancer Res. 2021 Feb 1;81(3):732-746. doi: 10.1158/0008-5472.CAN-20-1200. Epub 2020 Nov 12.PMID: 33184106. We are working on a manuscript with the clinical and laboratory correlates now that the data are complete.

We are also assessing 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) will be performed using a modified version of the Archer VariantPlex Solid Tumor assay (ArcherDx, Boulder CO). This next generation sequencing-based assay surveils for point mutations and insertions/deletions in 69 genes, with full exonic coverage for some genes and select exon and hotspot coverage for others. In addition we are performing gene expression assays with BioSpyder. From frozen sections we will be utilized for RPPA in laboratory of Dr. Chip Petricoin on frozen sections laser captured to enrich for tumor.. Dr. Richer is coordinating the molecular analyses for these two trials through our Pathology core (Dr. Adrie Van Bokhoven is director with Dr. Scott Lucia) and will oversee analyses of all of samples.

Clinical Aims:

The tumor specimens from the IIT Phase II Fulvestrant +/- Enzalutamide trial in the metastatic adjuvant and neoadjuvant setting will be analyzed as follows in this diagram.

This analysis uses a custom Archer VariantPlex that evaluates 60 gene hot spots for commonly mutated genes in cancer with our Colorado Molecular Correlates Laboratory (CMOCO) custom version assay. We are

particularly interested in mutations in the estrogen receptor alpha (ERalpha) and this assay covers detection of any mutation in exon 8 which covers N519 through the C-terminus V595 (based on refseq NM_000125) this includes the 3 most common ER mutations that arise in metastatic ER+ breast cancers treated with aromatase inhibitors (AI) and we also added the AR mutation hotspot that arises with enzalutamide during treatment of prostate cancer). The limit of detection for clinical cases is 10% allele frequency, but it often can detect down to 3.0%.

For the RPPA we are evaluating with Univ of CO sending Dr. Petricoin's lab the first set for phospho-protein analysis from frozen sections laser captured to enrich for tumor. "For reverse phase phospho-protein analysis (RPPA) we processing frozen sections to send to Dr. Petricoin's lab to perform the assay. When necessary, laser captured is performed to enrich for tumor. The first set includes 8 specimens from the metastatic trial (16-001). The next set will be the first batch from the pre-operative trial since this will give a clear idea of the changes induced by treatment since the primary tumors are not pretreated, while the metastatic disease has been. Also the biopsies and post-treatment specimens from the neoadjuvant trial are both from the primary, while for the metastatic trial the pre- and post-treatment specimens are often from biopsies of different accessible metastases.

We have performed mutation Archer VariantPlex assay to detect common mutations in the biopsies from the first 18 patients in the metastatic disease trial that had enough evaluable material in the core needle biopsy from a pretreatment core. Results indicated that of 18 tumors assayed, about half had mutations in the ESR1 exon 8 mutations which result in a constitutively active ERalpha because the ligand binding domain is in a position similar to as if it was bound to ligand. Additional mutations were found in ATM, SMAD4, TP53, PIK3CA, CDH1, and PTEN. Mutation results were published in **PMID: 33184106 and immunohistochemistry representative figures for ER, PR and AR protein as well**. All slides were scored by pathologist Sharon Sams. While it has been published that PR is higher in ER mutated tumors and cell lines, AR had not yet been examined at the protein level in patients with metastatic breast cancer with or without ER mutations.

What opportunities for training and professional development has the project provided?
he main graduate student that worked on this project was Valerie Barton who then obtained an NIH NRSA on work that stemmed from this grant. She defended her dissertation in April 2016 and obtained her doctorate from the University of Colorado Cancer Biology Graduate Program.

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.

Professional research assistant Nicole Spoelstra 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 mutltiplex staining for the tumor infiltrating lymphocytes and learned this technique at a week-long workshop in Boston when it first started.

Postdoctoral fellow Michelle M Williams has been working on preclinical aspects of AR in the ESR1 mutant ER+ disease. She was first author on **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 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.

How were the results disseminated to communities of interest?

During the last reporting period Dr. Richer gave the following lectures at national meetings. However, no grant funds were used to support the travel.

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."

April 2021* **American Society of Biochemistry and Molecular Biology** – Signaling in Breast and Ovarian Cancer Interest Group

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."

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

We presented posters virtually on the trial for metastatic disease at SABCS 2020, AACR 2020 and will do this year at SABCS: see abstract here:

Response of persistent metastatic ER+/Her2- breast cancer treated with fulvestrant plus enzalutamide

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Background: The clinical implications of the androgen receptor (AR), particularly in the context of aromatase inhibitor (AI) refractory metastatic breast cancer (MBC), are unclear. While AR is associated with more indolent primary tumors, in the absence of low estradiol/blocked ER, AR can exert a pro-survival signal. Thus, in a phase II trial of Fulvestrant (Fulv) plus Enzalutamide (Enza) in ER+/Her2- MBC (NCT02953860) we analyzed serial biopsies pre- and post-treatment.

Methods: Eligible patients were women with ECOG 0-2, ER+/Her2- MBC. Fulv at 500 mg IM days 1, 15, 29 and every 4 weeks thereafter and Enza at 160 mg po daily on a continual basis were administered. Biopsies were required at study entry and at ~4 weeks on therapy. The clinical benefit rate at 24 weeks (CBR24) was the primary endpoint for efficacy. We performed mutational analysis using a modified Archer VariantPlex Solid Tumor Assay to detect mutations in *ESR1* exon 8 and 67 other gene hotspots. We examined estrogen, progesterone, androgen and glucocorticoid receptor protein by IHC and multiplex for immune cells and PD-L1. Frozen cores were utilized to perform reverse phase protein array (RPPA) based protein pathway activation analysis of over 150 proteins from LCM-enriched tumor in baseline and post-treatment metastatic biopsies. Comparisons of Responders (progression free survival (PFS) equal to or longer than 24 weeks) and Non-Responders were performed using moderated t-tests on log2 transformed data.

Results: 32 patients were eligible, median age was 61 years (46-87), and 90.6% had visceral disease with an average of 4 prior non-hormonal therapies and 3 prior hormonal agents (including 37.5% with prior Fulv). PFS >24 weeks was observed in ~22% of patients treated with Fulv plus Enza, including 42% of those who had prior Fulv. When stratified by both AR and ER protein levels, median time to progression was 59 days (95% CI: 55 to Inf) when both targets were high (greater than or equal to 10%), but only 14 days (95% CI: 13 to Inf) when both were less than 10%. Metastases with *ESR1* mutations in the ligand binding domain had significantly higher levels of ER and PR protein than those with wild type *ESR1* ($p<0.05$), while AR did not significantly differ. ER significantly decreased following 5 weeks post Fulv plus Enza in a paired t-test ($p<0.003$). *ESR1* mutation positive metastases had significantly more T helper cells, T regulatory cells and macrophages than those with wild type *ESR1*. In contrast, those with *TP53* or *PIK3CA* mutations had higher CD8+ T cells, but also increased T regulatory cells compared to those WT for these genes. PD-L1 increased with treatment in all patients by paired t test ($p<0.03$). RPPA analysis indicated that activation of mTOR pathway proteins was associated with non-response to Fulv plus Enza and patients with *PIK3CA* and or *PTEN* mutated disease had a shorter progression free survival time following treatment, with the hazard of disease progression for participants with *PIK3CA* or *PTEN* mutated disease being 2.27 times (95% CI: 0.94 to 5.46) than without these mutations ($p=0.068$).

Conclusions: PFS >24 weeks was observed in 22% of patients treated with Fulv plus Enza, including 42% who had prior Fulv treatment, suggesting contribution of the anti-androgen. Response was significantly better when metastases were >10% for ER and AR. Poor response to Fulv plus Enza was significantly associated with mTOR pathway activation and patients with *PIK3CA* and or *PTEN* mutated metastases had a significantly shorter PFS. Mutation status also affected hormone receptor expression and immune infiltrates. The increase in PD-L1 protein following treatment with Fulv plus Enza warrants further pre-clinical investigation into whether the addition of anti-androgen can enhance efficacy of checkpoint inhibitor therapy in ER+ metastatic disease resistant to standard endocrine therapy approaches.

- **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 clinical, which will complete enrollment in the next couple of months. We will then finish the RPPA and gene expression data analysis for this trial.

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 the hormone receptors completely and may no longer be candidates for fulvestrant or any endocrine therapy.

- **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 may file a patent on the technology of staining for all of the hormone receptors on the same section and also on the results of the staining for all receptors in the mutant versus non-mutant disease.
  - **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.

5. CHANGES/PROBLEMS: Nothing to Report

Changes in approach and reasons for change

- Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.
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- **Actual or anticipated problems or delays and actions or plans to resolve them**

We found that AR protein expression in TNBC PDX models was decreasing even after two passages. However, we have now found that with androgen treatment, AR is re-expressed. We also recently found that ER+ PDX recapitulate the clinical samples in that those with ESR1 mutations express higher AR.

- **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: Journal publications. We have 3 new publications listed below in red.

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

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 gave the following lectures: with new this past year in red.

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?"

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*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."

*remote due to Covid19

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**. None
- **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."

- **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.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**
  Dr. Richer and her technician continue to analyze the molecular correlates from the study including gene expression, RPPA and immunohistochemstry in a blinded fashion. The technician Nicole Spoelstra does the immunostaining of biopsies from the clinical aims and pathologist scores them. They are all paid for now from Dr. Elias's partnering grant

- **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?** *Nothing to Report*

SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** Partnering PI, Dr. Anthony Elias has sent a separate report on the clinical progress.
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- **APPENDICES:** none