Title

Dual Therapy with Insulin-Like Growth Factor-I Receptor/Insulin Receptor (IGF1R/IR) and Androgen Receptor (AR) Antagonists Inhibits Triple-Negative Breast Cancer Cell Migration In Vitro

Journal

SPG biomed, 2(2)

Authors

Hamilton, Nalo
Márquez-Garbán, Diana
Rogers, Ben
et al.

Publication Date

2019

DOI

10.32392/biomed.69

Peer reviewed

Published: May 10th, 2019

Citation: Hamilton et al. (2019) Dual Therapy with Insulin-Like Growth Factor-I Receptor/Insulin Receptor (IGF1R/IR) and Androgen Receptor (AR) Antagonists Inhibits Triple-Negative Breast Cancer Cell Migration In Vitro. SPG BioMed 1(2).

Corresponding Author:
Nalo Hamilton
UCLA School of Nursing, Los Angeles, CA 90095 & UCLA Jonsson Comprehensive Cancer Center, Los Angeles, CA 90095
Email: nhamilton@sonnet.ucla.edu

RESEARCH ARTICLE

Dual Therapy with Insulin-Like Growth Factor-I Receptor/Insulin Receptor (IGF1R/IR) and Androgen Receptor (AR) Antagonists Inhibits Triple-Negative Breast Cancer Cell Migration In Vitro

Nalo Hamilton[1*], Diana Márquez-Garbán[2], Ben Rogers[3], David Austin[4], Kay Foos[5], Ashley Tong[6], Diana Adams[7], Jaydutt Vadgama[8], Mary-Lynn Brecht[9], Richard Pietras[10]

Author affiliations:
[1] UCLA School of Nursing, Los Angeles, CA 90095 & UCLA Jonsson Comprehensive Cancer Center, Los Angeles, CA 90095
[2] Department of Medicine, Division of Hematology-Oncology, UCLA David Geffen School of Medicine, Los Angeles, CA 90095 & UCLA Jonsson Comprehensive Cancer Center, Los Angeles, CA 90095
[3] UCLA Department of Biostatistics, Los Angeles CA 90095
[4] Molecular Screening Shared Resources (MSSR) UCLA California NanoSystems CNSI Los Angeles CA 90095
[5] UCLA College of Life Sciences, Department of Integrative Ecology and Evolutionary Biology and Physiology, Los Angeles, CA 90095
[6] UCLA College of Life Sciences, Department Integrative Biology and Physiology, Los Angeles, CA 90095
[7] Massachusetts Institute of Technology, Department of Bioengineering, Cambridge, MA 02139
[8] Charles Drew University School of Medicine and Science, Department of Medicine, Division of Cancer Research and Training, Los Angeles, CA 90059 & UCLA Jonsson Comprehensive Cancer Center, Los Angeles, CA 90095
[9] UCLA School of Nursing, Los Angeles, CA 90095
[10] Department of Medicine, Division of Hematology-Oncology, UCLA School of Medicine, Los Angeles CA 90095 & UCLA Jonsson Comprehensive Cancer Center, Los Angeles, CA 90095

Abstract

Background: Few targeted therapies are currently available for triple-negative breast cancers (TNBC) due to their lack of estrogen receptor-α and progesterone receptor expression and HER-2 amplification. In the clinic, TNBC is associated with a range of adverse biologic features

including a highly metastatic phenotype and early patient relapse, leading to poor clinical outcomes. Identifying effective treatment strategies to inhibit or prevent TNBC metastasis could help to improve patient treatment options and clinical outcomes.

Methods: Using immunohistochemistry, archival breast specimens from women with TNBC were screened for androgen receptor (AR), Slug and insulin-like growth factor-2 (IGF2) expression. The effects of IGF2 and combinations of insulin-like growth factor-1 receptor/insulin receptor (IGF1R/IR) and AR antagonists on TNBC cell migration was assessed in vitro using scratch wound assays, and cell death initiated by dual combinations was assessed using caspase assays. In parallel, gel electrophoresis and Western immunoblot assays were used to test effects of IGF1R/IR and AR antagonists on Slug expression. Finally, using the model of Response Additivity, drug synergy was assessed using different therapeutic treatment strategies.

Results: Based on modified Allred scoring results, IGF2, Slug and AR were identified in archival TNBC specimens. Further, IGF2 stimulated migration of TNBC cells MDA-MB-231 and HCC 1937 *in vitro*. Combination therapies of a) IGF1R/IR inhibitor BMS-754807 plus IGF1R inhibitor NVP-AEW807 or b) AR inhibitor enzalutamide together with either BMS-754807 or NVP-AEW807 led to inhibition of TNBC cell migration ($P<0.001$) and reduced expression of Slug. Additionally, combination treatments promote TNBC cell apoptotic activity *in vitro* ($P<0.005$). Significant synergy was detected ($CI < 1$) with enzalutamide plus NVP-AEW541 or BMS-754807 in BT549 and HCC 1937 cultures for cell migration *in vitro*; while combination of BMS-754807 plus NVP-AEW541 elicited antagonist effects ($CI > 1$) in BT549, MDA-MB-231 and HCC 1937 cultures.

Conclusion: These findings indicate that combinations of IGF1R/IR and AR antagonists can prevent TNBC migration, induce apoptosis and impact known mediators of metastasis, thus suggesting a potential for future application of such dual treatments to manage TNBC progression in the clinic.

Introduction

The majority of breast cancers (BC) are estrogen receptor-α (ERα) positive and often respond to endocrine therapy. In contrast, triple-negative breast cancers (TNBC), lacking expression of ERα and progesterone receptor (PR) and HER2 overexpression, cannot be managed with current targeted therapies. Accounting for almost half of all breast cancer deaths, TNBCs tend to occur in women younger than 40 years of age and exhibit aggressive behavior with a highly metastatic phenotype and a high incidence of recurrence after use of standard chemotherapy, often resulting in reduced patient survival [1-3]. Thus, the identification of novel therapeutic regimens to combat TNBC spread are urgently needed.

Insulin-like growth factor-2 (IGF2) is reported to play a role in cancer proliferation, differentiation and migration [4, 5]. IGF2 is overexpressed in TNBC, and a recent study demonstrates it is highly expressed in TNBC tumors from African-American and Latina women [6-8]. Thus, IGF2 may serve as a potential biomarker for TNBC treatment, and perturbation of IGF2-mediated signaling pathways may inhibit TNBC migration and spread. By interaction with specific tyrosine-kinase receptors, insulin-like growth factor-1 receptor (IGF1R) and insulin receptor isoform A (IR-A), IGF2 stimulates downstream actions via the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) AKT/protein kinase B (PKB) pathways to stimulate tumor proliferation [9]. A recent study highlights the significant anti-proliferative activities of IGF1R and IR antagonists in TNBC cells *in vitro* [10]. BMS-754807, an established IGF1R/IR inhibitor, and NVP-AEW541, an IGF1R inhibitor, exhibit anti-proliferative effects on IGF2-expressing TNBC cells, an action dependent in part on AKT activity. Of note, growth inhibitory effects were significantly enhanced when BMS-754807 and NVP-AEW541 were used in combination. Additionally, these two antagonists were each found to independently decrease the expression of androgen receptor (AR) in TNBC cells, suggesting potential cross-communication between IGF1R/IR and AR signaling pathways [10].

The androgen receptor (AR) is a member of the nuclear steroid receptor superfamily, and AR is expressed in 70-90% of all invasive breast cancers, often when tumor ER and PR are reportedly not detectable [11, 12, 13]. Notably, AR is independently reported to be expressed in 17% - 35% of TNBCs [14-16]. Overexpression of AR is associated with constitutive activation of IGF1R and AKT pathways in non-TNBCs [17, 18], and exposure of TNBC cells to the anti-androgen enzalutamide significantly reduces proliferation and invasion of the luminal AR TNBC subtype [19, 20]. Further, emerging reports indicate that proliferation of TNBC cells with basal-like and mesenchymal-like molecular subtypes are inhibited by either enzalutamide alone or in combination with IGF1R/IR antagonists [10, 21].

The epithelial-mesenchymal transition (EMT) is a critical component of tumor cell metastasis and invasion. As characterized by disruption of adhesion junctions and gain in mesenchymal markers, EMT is stimulated by several signal transduction pathways that converge in the activation of core transcription factors, such as Slug [22, 23]. Slug, a member of the SNAIL family, is aberrantly expressed in various tumor types and is able to modulate breast tumor invasion and metastasis [24]. This zinc-finger protein has been found to disrupt cell adhesion junctions by repressing the expression of E-cadherin [25] , and significant overexpression of Slug is found in breast tumors associated with lymph node metastasis [24]. EMT is further able to confer an aggressive phenotype associated with chemotherapy resistance [26].

In view of the dearth of treatment options for TNBC, its aggressive nature and high expression of IGF2, we have investigated the *in vitro* effects of IGF1R/IR and AR antagonists in combination on TNBC migration and apoptosis, as well as synergy among dual treatment strategies.

Methods

Cell Culture: Cell lines were obtained from the American Type Culture Collection (ATCC) and cultured according to ATCC recommendations. Briefly, cultures were maintained at 37°C in a 5%

CO_2 incubator. TNBC (ERα-/PR-/HER2-) MDA-MB-231, BT549 and HCC 1937 cells were cultured in RPMI 1640 media (ATCC) containing L-glutamine with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah), 100 I. U./ml penicillin and 100 µg/ml units streptomycin sulfate (Cellgro, Manassas, VA).

Immunohistochemistry (IHC): Overall, five archival TNBC specimens were available for this study. Retrospectively-collected and de-identified human tumor specimens were provided by the NCI-supported Cooperative Human Tissue Network (chtn.org). The malignant status of archival breast samples was confirmed by surgical biopsy/pathology and available follow-up data. All specimens were collected with appropriate institutional human subject protection committee approvals and patient consent. Formalin-fixed and paraffin-embedded tissue samples were stained using standard IHC protocols as before [27, 28 29]. Antibodies used included AR (1:25, Agilent M356201-2), Slug (1:50, Cell Signaling #9585), and IGF2 (1:100, AbCam). Appropriate controls were included to assess specificity and to validate each antibody including i) appropriate pre-immune control; ii) dose-dependent titration; iii) known positive and negative tissues; iv) use of specific peptide competitors; and v) other established approaches. Antigen expression was quantified as previously described [29-32].

Inhibition Assays: To assess the inhibitory effect of BMS-754807/NVP-AEW541, BMS-754807/enzalutamide and NVP-AEW541/enzalutamide combinations on Slug expression, TNBC cultures were grown in complete media to 75-80% confluence, then cultured for 24 hrs in phenol red-free RPMI media supplemented with 2% dextran-coated charcoal stripped fetal-bovine serum and the indicated combinations of either BMS-754807 (20µM; Selleckchem, Houston, TX), NVP-AEW541 (8µM, Selleckchem, Houston, TX) or enzalutamide (20µM; Selleckchem, Houston, TX). Total protein was isolated for Western immunoblot analysis using standard methods. Briefly, using RIPA buffer, total protein was isolated, and protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermofisher Scientific). Thirty micrograms (30µg) of total cell protein was resolved by 4-15% SDS-PAGE, transferred to a PVDF membrane and probed with antibodies directed against Slug (1:500, Cell Signaling #9585), with β-actin (1:2000, Santa Cruz Biotechnology, sc-47778) as a loading control.

Scratch Wound Assays: To assess the effect of IGF1R/IR and AR inhibitors on TNBC migration, 60,000 cells/well were seeded onto a 96-well ImageLock tissue culture plate and incubated overnight at 37°C in a 5% CO_2 incubator. To create precise wounds in each well, the WoundMaker (Essen Bioscience) was used. Dishes were washed according to the manufacture's protocol followed by the addition of 100µL of inhibitor-conditioned media. Plates were placed in an IncuCyte® S3 (Essen Bioscience) module and equilibrated for 10 minutes. As per the scanning schedule, images were obtained every 2 hrs to assess cell migration and wound confluence.

Caspase Assay: To evaluate the ability of inhibitor combinations to stimulate apoptosis, 5,000 cells/well were seeded on a 96-well plate in complete media and placed in the IncuCyte S3® module in an incubator at 37°C with 5% CO_2. Images were captured every 2 hrs using the IncuCyte S3® Live-Cell Analysis System. Once cultures reached 30% confluence, growth media was removed and replaced with inhibitor (BMS-754807/NVP-AEW541, BMS-754807/enzalutamide and NVP-AEW541/enzalutamide; BMS-754807 20µM, NVP-AEW541 8µM, enzalutamide 20µM)

conditioned media containing 1:1000 Caspase-3/7 Green (Essen Bioscience #4440) and IncuCyte Red Cytotoxicity Reagent (Essen Bioscience #4632). Following a 30-minute warming period, images were captured every 2hr over 4 days using the IncuCyte S3® Live-Cell Analysis System.

Synergy Assays: To evaluate the synergistic effects of combination treatments, TNBC cells cultured in 96-well plates at 60 -65% confluence were exposed to IGF1R/IR and AR inhibitors alone and in combination for 72 hrs. TNBC cell proliferation under these conditions was evaluated using CellTiter 96®Aqueous Non-radioactive Cell Proliferation Assay (20ul/well per the manufacturer's protocol; Promega). After 1 hr incubation at 37°C with 5% CO2, absorbance was assessed at 490 nm. Drug synergism was modeled using a Response Additivity approach as previously described[33]. For each drug combination, a Combination Index (CI) was calculated such that a value of CI<1 represents a synergistic relationship, while a value of CI>1 represents an antagonistic relationship. Assuming E_A and E_B are the effects of drug dose combinations A and B, respectively, and E_{AB} is the effect of using both drug doses A and B in combination, the Combination Index (CI) is defined as $CI=(E_A+E_B)/E_{AB}$. Effects of drug were modeled using dummy variables representing each drug and dose in multiple linear regression models with interactions terms representing synergistic/antagonistic relationships between drugs. Significance of drug synergy was determined using the *P* values associated with the interaction terms.

Statistical Analysis: GraphPad Prism software (www.graphpad.com) was used for statistical analysis. Unpaired student *t*-tests were used to determine data significance. P-values <0.05 were considered significant. Experiments were repeated at least three times.

Results

IGF2, AR and Slug expression in archival breast specimens: Relapse and distant spread for women with TNBC is significantly higher than that of other tumor types, and metastasis is made possible in part by epithelial-mesenchymal transition (EMT). In addition to IGF2, both Slug and AR have been associated with the process of tumor metastasis. Consequently, the expression of IGF2, AR and Slug in malignant TNBC breast tissue was assessed by immunohistochemistry in archival breast tissue from five patients with TNBC specimens and compared with their expression in neighboring normal tissue (Figure 1).

In accord with earlier reports, significant expression of IGF2 is found in normal and TNBC tissue [6, 7, 34], and Slug and AR are each notably expressed in TNBCs.

IGF2 stimulates TNBC cell migration: Since IGF2 is highly expressed in aggressive TNBC tumors [6, 7, 35], we assessed the effects of IGF2 on TNBC migration *in vivo*. Our studies show IGF2, in the absence of other growth factors and fetal bovine serum, is able to stimulate migration of MDA-MB-231 cells, increasing wound confluence by up to 50% (Figure 2A, P<0.0001).

Notably, in HCC 1937 cultures, IGF2 similarly stimulated cell migration, increasing the number of cells in the wound tract from essentially zero to 100%, resulting in complete wound closure (Figure 2B, P<0.0001). These data indicate that IGF2, in the absence of FBS, has the ability to stimulate migration of TNBC cells *in vitro*.

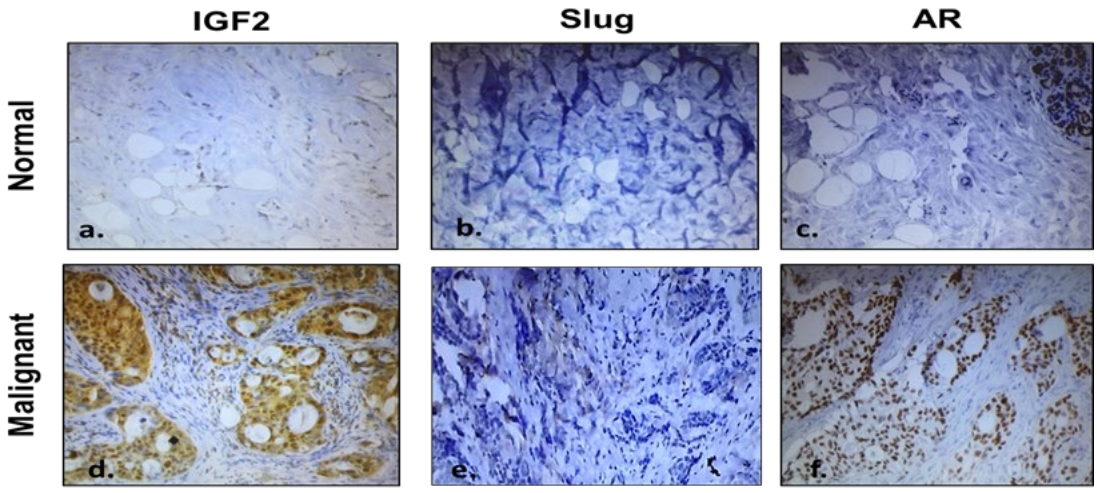

Figure 1. IGF2, Slug and AR are detected in archival TNBC breast tissue. Retrospectively-collected and de-identified breast tissue specimens from women with breast cancer along with adjacent normal tissue was obtained from the NIH CHTN. Immunohistologic assays were used to evaluate tissue samples for IGF2, Slug and AR expression (see Methods). Expression of IGF2 (a & d) Slug (b & e) and AR (c & f) were evaluated and visualized using Evos xl Core Microscope (200x). Representative images are presented.

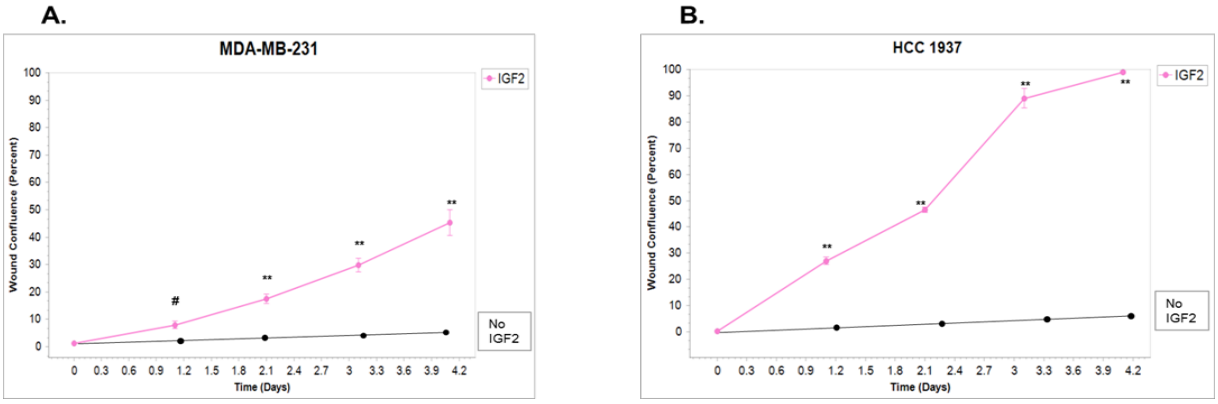

Figure 2. IGF2 stimulates TNBC cell migration. MDA-MB-231 (**2A**) and HCC 1937 (**2B**) TNBC cell lines were cultured in complete media in 96-welled plates. In each well 60,000 cells were plated. Scratch wounds were made using Unique WoundMaker™. Wound images were collected every 2 h for 5 days using the IncuCyte S3 Live-Cell Analysis System. Media containing IGF2 (100 ng/ml) was refreshed every 12 hrs. Error bars = SEM; N=5 **P<0.0001; #P< 0.005.

Combinations of IGF1R/IR and AR antagonists inhibit TNBC cell migration: IGF2 stimulates its downstream effects by binding to IGF1R/IR [7, 35]. In addition, we have also discovered that IGF1R/IR antagonists can significantly reduce androgen receptor (AR) expression in TNBC cells[10]. This relationship may be important because independent clinical studies associate AR expression with aggressive, metastatic BCs, and AR is notably expressed and/or enriched in specific subtypes of TNBC[36] (Figure 1).

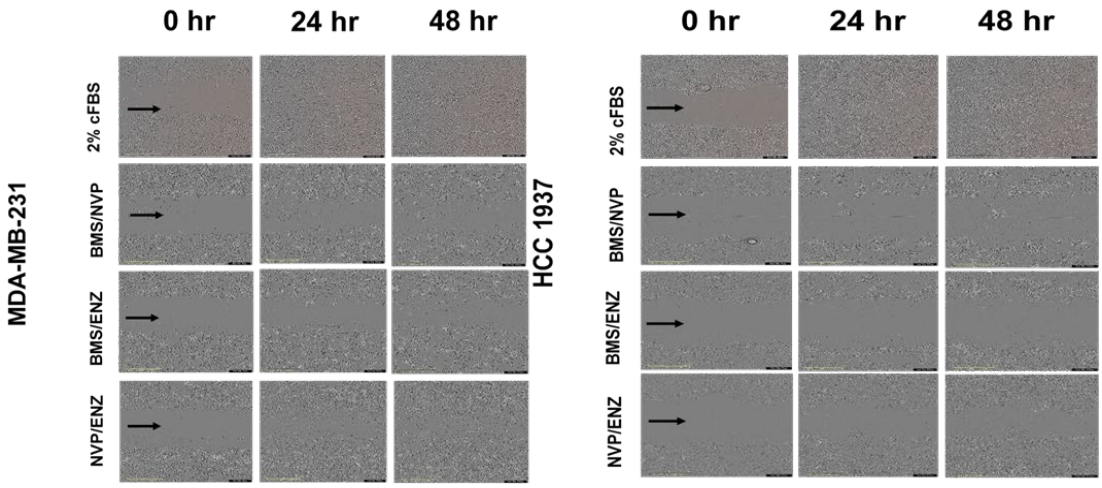

Figure 3A. Inhibition of TNBC migration by combinations of IGF1R/IR and AR antagonists. MDA-MB-231 and HCC 1937 TNBC cell lines were seeded in complete media in 96-well plates. In each well, 60,000 cells were plated. Scratch wounds were made using Unique WoundMaker™. Wound images were collected every 2hr for 48hr using the IncuCyte S3 Live-Cell Analysis System. In 2% cFBS, phenol-free RPMI media containing a combination of IGF1R/IR and AR inhibitors, cell migration was inhibited. Magnification 100x.

To test the effect of IGF1R/IR and AR antagonist combinations on TNBC cell migration, we used BMS-754807, a dual IGF1R/IR antagonist, NVP-AEW541, an IGF1R antagonist, and enzalutamide, an AR antagonist (Figure 3A). In comparison to appropriate controls, each inhibitor combination significantly inhibited cell migration to prevent wound closure (Figure 3B & 3C). In MDA-MB-231 cultures, the greatest inhibitory effect was achieved with combinations of BMS-754807/NVP-AEW541 and BMS-754807/enzalutamide (Figure 3B, $P<0.0001$). The combination of NVP-AEW541/enzalutamide also prevented cell migration but not to the same extent as combinations containing BMS-754807 ($P<0.001$). In HCC 1937 cultures, the combination of BMS-754807/enzalutamide had the greatest inhibitory effect against cell migration, resulting in only 12% wound closure followed by BMS-754807/NVP-AEW541 with wound closure of 18% ($P<0.0001$). Although the combination of NVP-AEW541/enzalutamide significantly inhibited cell migration, cells were able to migrate enough to close the wound to 60% (Figure 3C, $P<0.001$). Thus, these data demonstrate that combinations of IGF1R/IR and AR antagonists prevent TNBC cell migration, significantly reducing wound confluence.

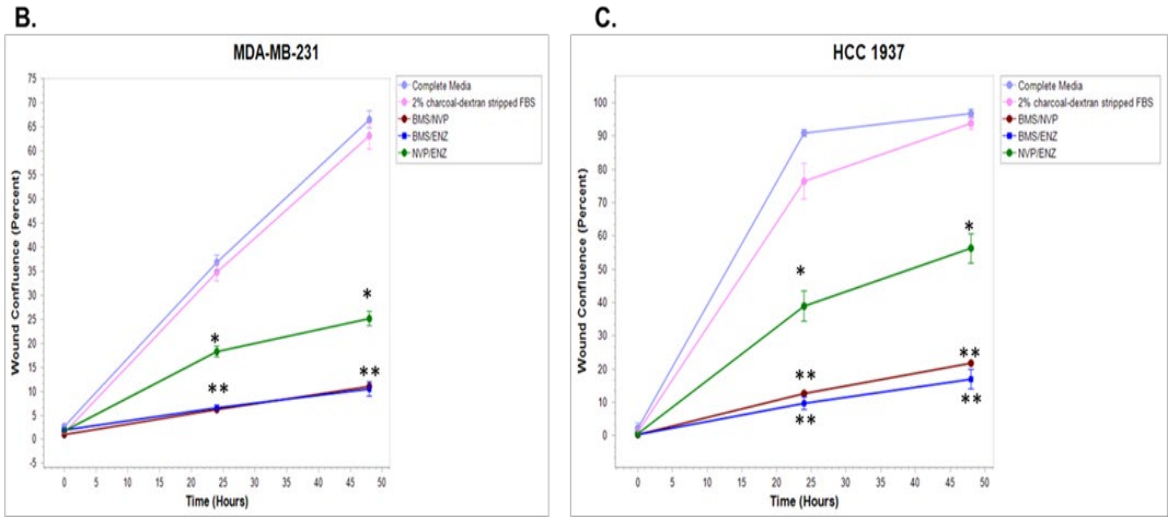

Figure 3. Inhibition of TNBC migration by combinations of IGF1R/IR and AR antagonists. 3B & 3C. Graphical representation of MDA-MB-231 and HCC 1937 cell migration under treatment conditions with IGF1R/IR/AR inhibitors. BMS/NVP = BMS-754807 (20uM) and NVP-AEW541 (8uM); BMS/ENZ=BMS-754807 (20uM) and enzalutamide (20uM); NVP/ ENZ= NVP-AEW541 and enzalutamide. **P<0.0001, *P<0.001 N=6.

Data from Western immunoblot analysis further indicate that combinations of BMS-754807/NVP-AEW541 and BMS-754807/enzalutamide also reduce the expression of Slug, a known mediator of EMT (Figure 4).

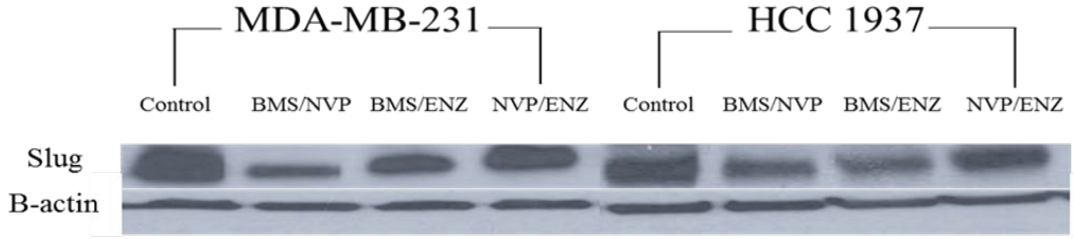

Figure 4. Effect of IGF1R/IR and AR inhibitor combinations on Slug. TNBC cultures MDA-MB-231 and HCC 1937 were seeded and grown to 70% confluence and then cultured in media containing IGF1R/IR/AR inhibitor combinations for 24hr. Proteins were separated and transferred to PVDF membranes that were probed for Slug (1:500, Cell Signaling #9585) following manufacturer recommendations. Image is a representative of three independent assays. BMS/NVP = BMS-754807 (20uM) and NVP-AEW541 (8uM); BMS/ENZ=BMS-754807 (20uM) and enzalutamide (20uM); NVP/ ENZ= NVP-AEW541 and enzalutamide.

Together, these data suggest that combinations of IGF1R/IR/AR antagonists effectively reduce TNBC cell migration at least in part by reducing the expression of Slug and potentially other regulators of EMT.

IGF1R/IR- and AR antagonist combinations stimulate apoptosis in TNBC cells: There is a therapeutic advantage in using drug combinations that inhibit cell metastasis while concurrently stimulating apoptosis, ultimately resulting in improved antitumor therapeutic efficacy. Thus, we assessed the ability of IGF1R/IR and AR combinations to stimulate apoptosis in TNBC cultures *in vitro* (Figure 5A).

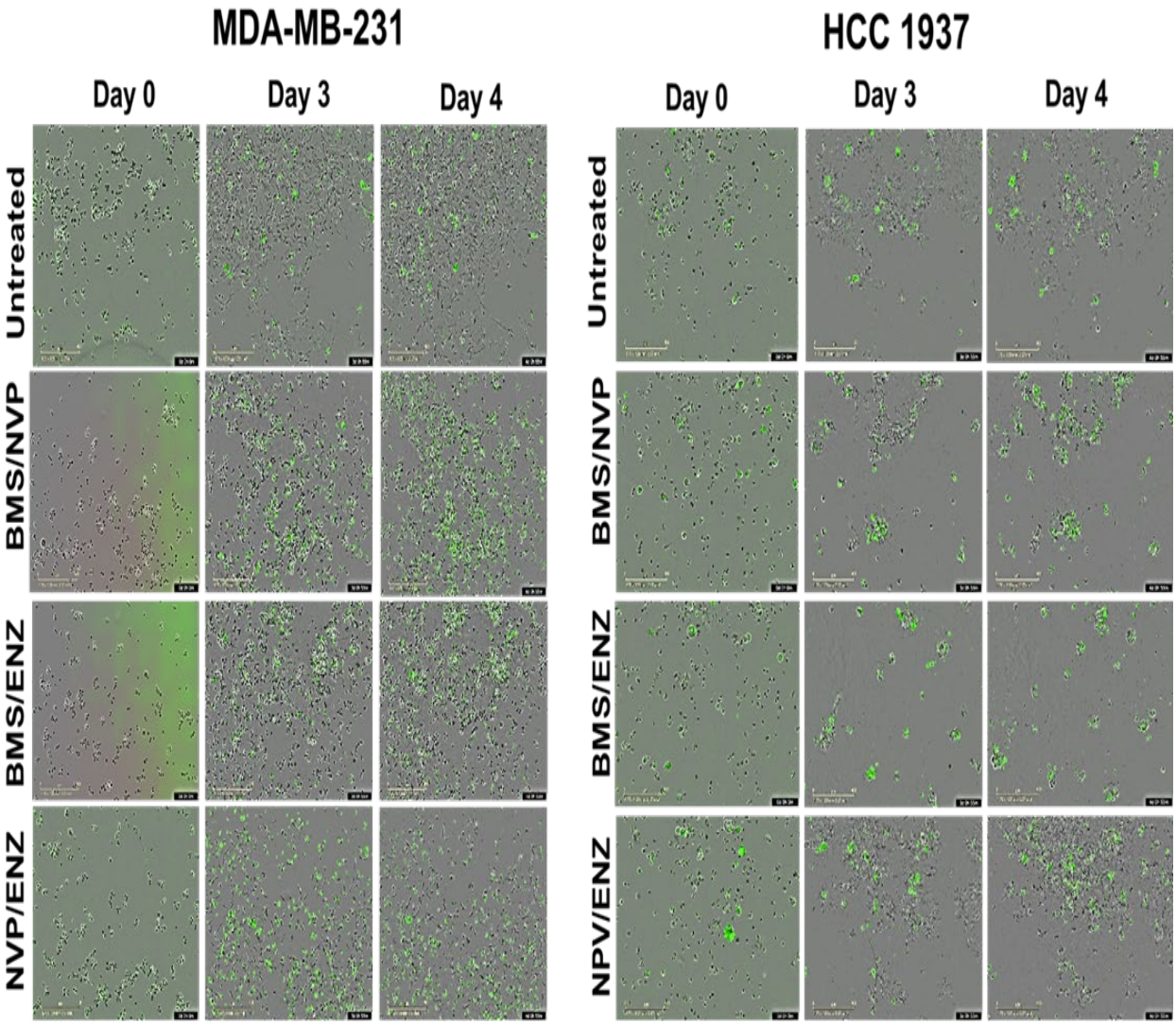

Figure 5A. IGF1R/IR and AR combinations stimulate caspase activity in TNBC cell lines. MDA-MB-231 and HCC 1937 TNBC cell lines were cultured in complete media in 96-welled plates. In each well 5,000 cells were plated. Images were captured every 2 hours using the IncuCyte S3 Live-Cell Analysis System. Once cultures reached 30% confluence growth media was removed and replaced with inhibitor (BMS-754807 20 uM, NVP-AEW541 8 uM, enzalutamide 20 uM) conditioned media containing 1:1000 Caspase-3/7 Green (Essen Bioscience #4440) and IncuCyte Red Cytotoxicity Reagent (Essen Bioscience # 4632). Following a 30 minute warming period, images were captured every 2 hours over 4 days using the IncuCyte S3 Live-Cell Analysis System. Magnification 100X.

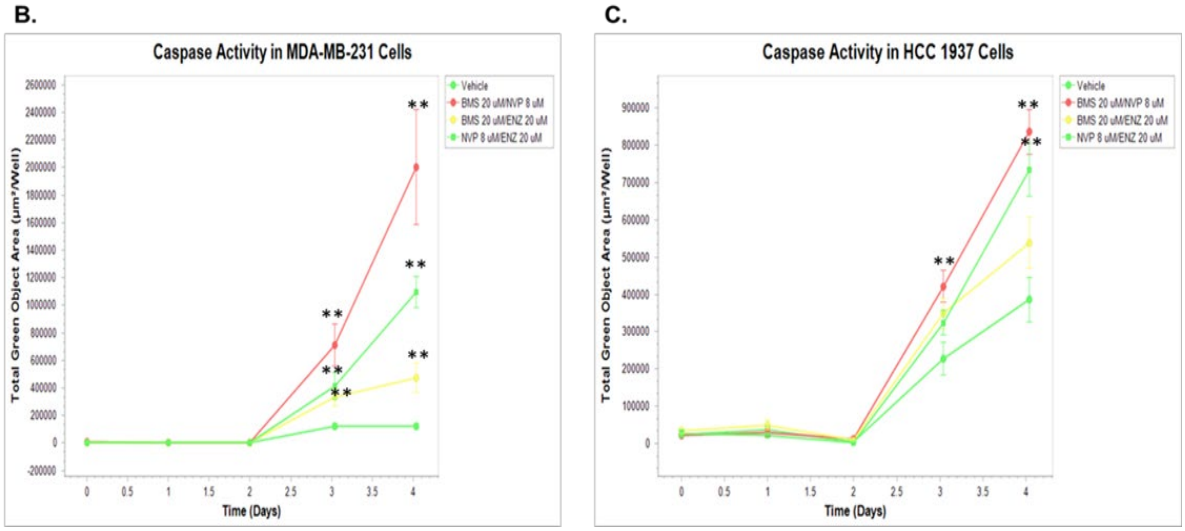

Figure 5. Combination of IGF1R/IR and AR inhibitors stimulate apoptosis TNBC cells. Graphical representation of caspase activity in MDA-MB-231 **(5B)** and HCC 1937 (**5C**) cultures under treatment conditions with IGF1R/IR/AR inhibitors. BMS/NVP = BMS-754807 (20uM) and NVP-AEW541 (8uM); BMS/ENZ=BMS-754807 (20uM) and enzalutamide (20uM); NVP/ ENZ= NVP-AEW541 and enzalutamide.

In MDA-MB-231 cultures, all drug combinations significantly stimulated caspase 7 activity at 72 hours ($P < 0.005$, Figure 5B). At 96 hours, the greatest residual apoptotic effect was detected with BMS-754807/NVP-AEW541 (P=0.0011; Fig. 5B) followed by the combination of NVP-AEW541/enzalutamide (P=0.0001; Figure 5B). The stimulation of apoptosis varied among treatment combinations and time point in HCC1937 cultures. In these cells, the combination of BMS-754807/NVP-AEW541 stimulated apoptosis at a significant level at 72 and 96 hours (P=0.0099 and P=0.0003). However, a significant apoptotic effect of NVP-AEW541/enzalutamide were not apparent until 96 hours (Figure 5C: P =0.0036), while the combination of BMS-754807/enzalutamide had no significant effect at any time-point. Here combinations of IGF1R/IR/AR inhibitors stimulated apoptosis in MDA-MB-231 cells but had variable effects in HCC 1937 cells. The variance in cellular response observed among cell lines may point to biological diversity and/or other cofounders.

Synergistic and Antagonist effects of IGF1R/IR and AR inhibitors in TNBC cell cultures: Due to the combinatorial effects of BMS-754807, NVP-AEW541 and enzalutamide *in vitro*, we wanted to determine whether these effects were synergistic in nature. Using the lowest concentrations that reduced cell proliferation by at least 50% (data not shown), TNBC cultures were exposed to 10µM BMS-754807, NVP-AEW541 at 5µM and 10µM concentrations, and 10µM of enzalutamide alone and in combination. We used the effect-based model of Response Additivity and calculated the Combination Index (CI) for each combination [33, 37, 38]. In BT549 cells, that express robust levels of AR, combinations of enzalutamide with BMS-754807 and NVP-AEW541, at either 5µM or 10µM, produced significant synergistic effects with CI < 1 (Table 1).

Table 1. Individual effects and combined effects of drug combinations on BT 549 cell lines (estimate, standard error).

Drug A	Drug B	E_A	E_B	Interaction	E_{AB}	CI	p
ENZ 10	NVP 5	.36 (.05)	-.59 (.05)	-.32 (.07)	-.54	.42	<.0001
ENZ 10	NVP 10	.36 (.05)	-.91 (.05)	-.34 (.07)	-.88	.62	<.0001
ENZ 10	BMS 10	.36 (.05)	-.71 (.05)	-.33 (.07)	-.68	.52	<.0001
NVP 5	BMS 10	-.59 (.05)	-.71 (.05)	.41 (.07)	-.89	1.47	<.0001
NVP 10	BMS 10	-.91 (.05)	-.71 (.05)	.72 (.07)	-.90	1.80	<.0001

A similar effect is observed in HCC 1937 cultures with the CI ranging from 0.79 - 0.92 (Table 2), whereas in MDA-MB-231 cultures, the only dual treatment combination to produce a synergistic CI < 1 was 10µM enzalutamide/10µM BMS-7548047 (Table 3). In all cell lines, the combination of BMS-754807/NVP-AEW541 consistently yielded a significant CI >1 indicating an apparent antagonist relationship between the two drugs (Tables 1, 2 & 3) despite the finding that these two different IGF1R antagonist drug combinations were effective in blocking TNBC cell migration *in vitro* (Figure 3).

Table 2. Individual effects and combined effects of drug combinations on HCC 1937 cell lines (estimate, standard error).

Drug A	Drug B	E_A	E_B	Interaction	E_{AB}	CI	p
ENZ 10	NVP 5	.11 (.05)	-.63 (.05)	-.05 (.08)	-.56	.92	.5264
ENZ 10	NVP 10	.11 (.05)	-1 (.05)	-.14 (.08)	-1.02	.87	.0747
ENZ 10	BMS 10	.11 (.05)	-.5 (.05)	-.1 (.08)	-.49	.79	.1732
NVP 5	BMS 10	-.63 (.05)	-.5 (.05)	.19 (.08)	-.94	1.20	.0124
NVP 10	BMS 10	-1 (.05)	-.5 (.05)	.43 (.08)	-1.07	1.40	<.0001

Table 3. Individual effects and combined effects of drug combinations on MDA-MB- 231 cell lines (estimate, standard error).

Drug A	Drug B	E_A	E_B	Interaction	E_{AB}	CI	p
ENZ 10	NVP 5	.04 (.02)	-.32 (.02)	.01 (.03)	-.27	1.03	.8124
ENZ 10	NVP 10	.04 (.02)	-.34 (.02)	.01 (.03)	-.29	1.01	.8587
ENZ 10	BMS 10	.04 (.02)	-.1 (.02)	-.01 (.03)	-.07	.78	.6965
NVP 5	BMS 10	-.32 (.02)	-.1 (.02)	.1 (.03)	-.32	1.32	.0035
NVP 10	BMS 10	-.34 (.02)	-.1 (.02)	.11 (.03)	-.32	1.36	.0015

Discussion

At diagnosis, only 15% of women have TNBC, yet women with TNBC continue to account for almost 50% for all breast cancer deaths[39, 40]. All women with metastatic TNBC will die of their disease, and fewer than 30% of women live 5 years after their diagnosis[41, 42]. This health disparity may be the result of unique biologic underpinnings. Although heterogeneous in nature, TNBCs tend to exhibit

aggressive growth, often with early relapse and metastasis after initial therapies. In this study, we document expression of IGF2, Slug and AR in archival breast tissue specimens from patients with malignant breast cancers. IGF2, a secreted growth factor, has high levels of expression in TNBCs and neighboring stromal cells [7]. In comparison to TNBC tumors from Caucasian women, IGF2 is expressed at significantly higher levels in TNBC tumors from African American and Latina women, populations that have the worst breast cancer outcomes[8]. Expression of Slug, an EMT regulator and inhibitor of DNA repair, is reported to correlate with inhibition luminal development of mammary epithelial cells (MEC), increased cancer stem cells (CSCs) levels and lymph node metastasis[24, 43], thereby identifying Slug as a biological marker for reduced patient survival. Additionally, there are several studies that associate AR expression with more aggressive breast tumor behavior[14, 15, 36, 44]. Similarly, results of this study suggest that expression of IGF2, Slug and AR associate with TNBC progression, but future studies using specimens from a larger and more diverse populations of TNBC patients are needed to confirm the relationship between these selected biomarkers and TNBC spread.

TNBC mortality is largely due to the distant metastatic spread of tumor cells. Previously, we reported that over-expression of IGF2 in archival TNBC tissue and its capability to activate IGF1R/IR signaling promoted TNBC proliferation[7]. In addition to stimulating TNBC proliferation, the current findings show the independent ability of IGF2 to stimulate TNBC migration. Thus, the overexpression of IGF2 in TNBC appears to foster an environment promoting both TNBC cell proliferation and tumor cell migration. Together, these findings suggest that IGF2 is a potential biomarker for aggressive, metastatic TNBCs.

IGF2 binds to both IGF1R and IR receptor tyrosine kinases that activate downstream signaling pathways for cell proliferation, apoptosis and migration[45]. The vast majority of TNBC cells express IGF1R, and binding of IGF1 or IGF2 to IGF1R is known to stimulate downstream effects that enable TNBC cells to proliferate and avoid programmed cell death[46, 47]. Hence, stimulation of TNBC apoptosis by using combinations of BMS-754807/ NVP-AEW541, BMS-754807/enzalutamide and NVP-AEW541/enzalutamide represent potential novel strategies to promote cell death in TNBC cells. Our findings add to previous studies demonstrating the induction of apoptosis with treatments containing BMS-754807 or NVP-AEW807 in TNBC and ER-positive breast tumor cells, as well as in synovial sarcomas and undifferentiated pleomorphic sarcomas[45, 48-52]. Further, constitutively active IGF1R promotes xenograft growth and EMT through stimulation of SNAIL[53]. In fact, the stimulation of the RAS/RAF or PI3K/AKT signaling pathways via IGF1R leads to the activation of several known EMT modulators[54]. The drug combinations presented in this study could potentially be future therapeutic options that stimulate TNBC cell apoptosis while simultaneously inhibiting mediators responsible for TNBC metastasis.

The EMT process may be a central component of TNBC heterogeneity and plasticity[55, 56]. As cells progress through the process of EMT, cell polarity is lost as well as adhesive junctional associations allowing for cell metastasis and invasion. E-cadherin is normally an important junctional component, and during EMT E-cadherin expression is significantly reduced, at least in part by the transcription factor Slug[57, 58]. The binding of Slug to E-box sequences in the promotor region of E-cadherin represses transcriptional activity and causes significant reduction of E-cadherin [24, 25, 59]. In similar fashion, Slug inhibits the expression of *BRCA2*, a necessary component of the DNA repair

complex[60]. Thus, the inhibitory effects of Slug on DNA repair and cell association permit TNBC development, progression and metastasis. Cevenini *et al.* detail IGF1R signaling pathways that stimulate known EMT modulators including Slug in breast cancers[54]. Thus, the reduction of Slug expression by BMS-754807/NVP-AEW541 and BMS-754807/enzalutamide drug combinations may potentially modulate Slug expression at both DNA and protein levels. Potentially, these inhibitor combinations may also regulate other EMT modulators such as Twist, ZEB1 and /or Snail to reduce TNBC migration and spread to improve overall patient survival.

Due to the complex nature of TNBC and the recognition of multiple drivers of tumor progression, it is important to consider the implementation of combination treatment modalities to stop disease spread. Advantages of combination therapies include better antitumor efficacy, less toxicity and reduced incidence of drug resistance. Previous investigations into targeted dual therapies for TNBC offer evidence for the IGF1R pathway as a promising therapeutic target[5, 10, 45, 48, 52, 61-64]. However, randomized trials have yet to show a significant clinical benefit by targeting the IGF1R pathway using IGFR1 inhibitors alone or combined with standard chemotherapeutics[65, 66]. This finding may be due, in part, to a lack of rational combination therapy regimens.

IGF2 achieves its biologic effects in large part via binding to IGF1R/IR, a signaling pathway that appears to cross-communicates with the AR signaling pathway[17, 67,17, 44, 68, 69]. Several reviews document the role of AR in breast cancer, the promise of AR targeting in the clinic and the potential of AR as a biomarker in TNBC[70-74]. Controversy surrounding the exact role of AR in TNBC, as some studies report that patients with AR-negative TNBCs have worse disease-free survival than those patients with AR-positive TNBCs, while other studies are unable to demonstrate a prognostic role for AR[75-77]. Such varied results may be attributable to the expression of bioactive AR splice variants within TNBCs[78]. In prostate cancer, the PI3K pathway is key to AR modulation, and the same pathway appears to be requisite for AR regulation in breast cancer[79, 80]. Additionally, cross-communication exists between ER and AR signaling, two pathways that are regulated by IGF1R[10, 35, 72,81, 82].

To date, little evidence exists evaluating dual IGF2/IGFR and AR-targeted therapy in TNBC. Thus, we evaluated a unique drug combination strategy targeting IGF2/IGFR-induced signaling and its interaction with AR in TNBC as an intervention to stop TNBC progression[10]. The current findings show that IGF1R/IR/-AR inhibitor combinations of BMS-754807/NVP-AEW541, BMS-754807/enzalutamide and NVP-AEW541/enzalutamide significantly reduce TNBC migration and also stimulate TNBC cell apoptosis. Notably, in support of previous reports, dual therapies containing enzalutamide demonstrate these effects even in TNBC cells expressing little AR protein[19, 83, 84].

Our combination treatments proved effective in the inhibition of TNBC cell migration and in the induction of apoptosis. To determine whether the combinatorial effect (E_{AB}) was greater than that achieved with either drug alone (E_A & E_B), we used the effect-based model of Response Additivity [33]. The combination index, calculated as: $CI = E_A + E_B / E_{AB}$ and outlined by the Chou-Talalay method, allows a standard measure of combination effects[33, 38]. In the conditions presented in this paper, dual IGF1R/IR-AR treatment combinations had both synergistic and antagonistic effects. In BT549 and HCC 1937 cell cultures, significant synergy was achieved with drug combinations containing

an IGF1R antagonist and enzalutamide, indicating that in both high- and low-AR expressing cell lines these dual combinations inhibit cell migration and stimulate apoptosis. Conversely, the combination of BMS-754807 with NVP-AEW541 demonstrated antagonism across all cell lines assessed. Nonetheless, this combination did significantly induce cell apoptosis and inhibit TNBC cell migration in each TNBC cell line. In independent studies, a synergistic drug effect was obtained with NVP-AEW541 combined with erlotinib or imatinib[85, 86] and when BMS-754807 was used in combination with tamoxifen, letrozole and chemotherapy[45, 48, 52]. However, antagonistic effects were found when May *et al.* used NVP-AEW541 in combination with BGT226, a dual PI3K/mTOR inhibitor, to combat growth and migration of undifferentiated pleomorphic sarcoma cell[51]. It is possible that the synergy calculations in the combination of BMS-754807 and NVP-AEW541 in the current study may be skewed because of non-overlapping toxicity in their mechanism of action, with both drugs impacting IGFR1, other cellular process that result in reduced TNBC migration. To fully understand the complex relationship among the promising combinations of BMS-754807 or NVP-AEW541 with enzalutamide in TNBC, it is clear that further large scale studies using *in vivo* tumor metastasis models will be needed. However, the data presented here demonstrate significant promise of these novel combinations as potential therapeutic options for TNBC.

Conclusions

TNBC patient mortality is disproportionately high compared to all other breast cancer subtypes. Currently, chemotherapy is one of the only standardized treatments for TNBC, and targeted therapies are not available for patients afflicted with this aggressive form of breast cancer. Increased IGF2 levels by autocrine or paracrine pathways in TNBC cells leads to IGF1R/IR signaling. The downstream effects of IGF2/IGF1R/IR association include the activation or upregulation of AR and the EMT mediator Slug, thereby resulting in poor patient outcome due to enhanced tumor proliferation and metastasis (Figure 6)[17, 54, 87].

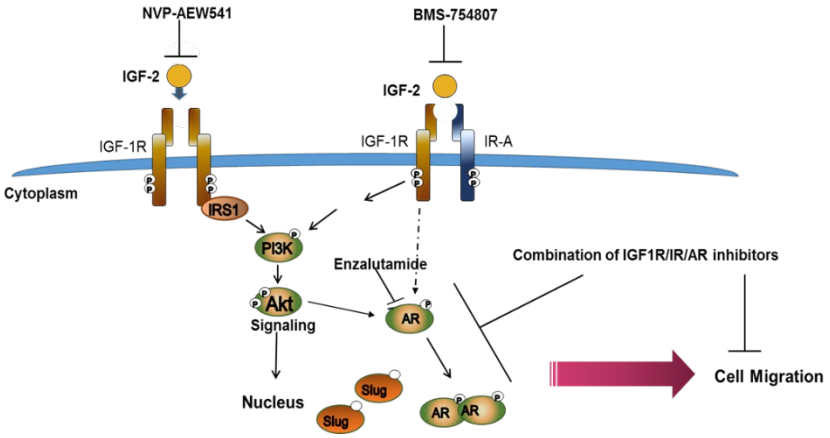

Figure. 6: Schematic of IGF2 stimulatory effects and inhibitory effects of dual IGF1R/IR-AR antagonist combination treatments in TNBC cells. The ability of IGF2 to stimulate downstream signaling via IGF1R and IR homo- and heterodimers is shown. IGF2-induced downstream stimulation of AKT in turn promotes activation of AR for translocation to the nucleus to promote the transcription of specific genes, such as IGF1R, that play a role in promoting TNBC cell proliferation and migration. These several IGF2-induced processes may be inhibited by combinations of NVP-AEW541, BMS-754807 and enzalutamide.

Thus, inhibition of IGF2 stimulated signaling represents a unique treatment methodology with therapeutic potential. Of importance, the IGFR signaling axis is also implicated to play a critical role in promoting an immunosuppressive tumor microenvironment, suggesting that combinations of IGFR inhibitors with emerging immune checkpoint inhibitor therapies may contribute further to blockade of TNBC progression[88, 89]. Combination therapies with BMS-754807 or NVP-AEW541 combined with enzalutamide may present new therapeutic options for TNBC, especially for tumors with high IGF2 expression. This unique co-targeting approach to stop TNBC migration and metastasis could potentially improve patient survival going forward.

Acknowledgements

Support. This work was supported by California Breast Cancer Research Program Grant #24IB-0053, Robert Wood Johnson Foundation Grant #69352, NIH/NCI U54 CA14393 Charles Drew University (CDU) School of Medicine/University of California at Los Angeles (UCLA) Jonsson Comprehensive Cancer Center Partnership and Tower Cancer Research Foundation- Jessica M. Berman Breast Cancer Research Award.

Conflict of Interest. Dr. Pietras has been a consultant with Astra-Zeneca, Pfizer and Genentech. The remaining authors deny any conflict of interest.

References

1. Carey, L. A.; Dees, E. C.; Sawyer, L., et al. The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes, *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2007, *13,* 2329-2334.

2. Reis-Filho, J. S.; Tutt, A. N. Triple negative tumours: a critical review, *Histopathology*. 2008, *52,* 108-118.

3. Bauer, K. R.; Brown, M.; Cress, R. D.; Parise, C. A.; Caggiano, V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry, *Cancer*. 2007, *109,* 1721-1728.

4. Singh, S. K.; Moretta, D.; Almaguel, F.; Wall, N. R.; De Leon, M.; De Leon, D. Differential effect of proIGF-II and IGF-II on resveratrol induced cell death by regulating survivin cellular localization and mitochondrial depolarization in breast cancer cells, *Growth factors*. 2007, *25,* 363-372.

5. Mancini, M.; Gariboldi, M. B.; Taiana, E., et al. Co-targeting the IGF system and HIF-1 inhibits migration and invasion by (triple-negative) breast cancer cells, *Br J Cancer*. 2014, *110,* 2865-2873.

6. Kalla Singh, S.; Tan, Q. W.; Brito, C.; De Leon, M.; De Leon, D. Insulin-like growth factors I and II receptors in the breast cancer survival disparity among African-American women, *Growth*

hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society. 2010, *20,* 245-254.

7. Hamilton, N.; Marquez-Garban, D.; Mah, V., et al. Biologic roles of estrogen receptor-beta and insulin-like growth factor-2 in triple-negative breast cancer, *BioMed research international*. 2015, *2015,* 925703.

8. Austin, D.; Hamilton, N.; Elshimali, Y.; Pietras, R.; Wu, Y.; Vadgama, J. Estrogen receptor-beta is a potential target for triple negative breast cancer treatment, *Oncotarget*. 2018, *9,* 33912-33930.

9. Lodhia, K. A.; Tienchaiananda, P.; Haluska, P. Understanding the Key to Targeting the IGF Axis in Cancer: A Biomarker Assessment, *Frontiers in oncology*. 2015, *5,* 142.

10. Hamilton, N.; Austin, D.; Marquez-Garban, D., et al. Receptors for Insulin-Like Growth Factor-2 and Androgens as Therapeutic Targets in Triple-Negative Breast Cancer, *Int J Mol Sci*. 2017, *18*.

11. Hu, R.; Dawood, S.; Holmes, M. D., et al. Androgen receptor expression and breast cancer survival in postmenopausal women, *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2011, *17,* 1867-1874.

12. Park, S.; Koo, J.; Park, H. S., et al. Expression of androgen receptors in primary breast cancer, *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*. 2010, *21,* 488-492.

13. Lea, O. A.; Kvinnsland, S.; Thorsen, T. Improved measurement of androgen receptors in human breast cancer, *Cancer research*. 1989, *49,* 7162-7167.

14. Gasparini, P.; Fassan, M.; Cascione, L., et al. Androgen receptor status is a prognostic marker in non-basal triple negative breast cancers and determines novel therapeutic options, *PloS one*. 2014, *9,* e88525.

15. Choi, J. E.; Kang, S. H.; Lee, S. J.; Bae, Y. K. Androgen receptor expression predicts decreased survival in early stage triple-negative breast cancer, *Ann Surg Oncol*. 2015, *22,* 82-89.

16. Mrklic, I.; Pogorelic, Z.; Capkun, V.; Tomic, S. Expression of androgen receptors in triple negative breast carcinomas, *Acta histochemica*. 2013, *115,* 344-348.

17. Rechoum, Y.; Rovito, D.; Iacopetta, D., et al. AR collaborates with ERalpha in aromatase inhibitor-resistant breast cancer, *Breast cancer research and treatment*. 2014, *147,* 473-485.

18. Lee, S. H.; Johnson, D.; Luong, R.; Sun, Z. Crosstalking between androgen and PI3K/AKT signaling pathways in prostate cancer cells, *The Journal of biological chemistry*. 2015, *290,* 2759-2768.

19. Barton, V. N.; Christenson, J. L.; Gordon, M. A., et al. Androgen Receptor Supports an Anchorage-Independent, Cancer Stem Cell-like Population in Triple-Negative Breast Cancer, *Cancer research*. 2017, *77,* 3455-3466.

20. Anestis, A.; Sarantis, P.; Theocharis, S., et al. Estrogen receptor beta increases sensitivity to enzalutamide in androgen receptor-positive triple-negative breast cancer, *Journal of cancer research and clinical oncology*. 2019, DOI 10.1007/s00432-019-02872-9.

21. Lehmann, B. D.; Jovanovic, B.; Chen, X., et al. Refinement of Triple-Negative Breast Cancer Molecular Subtypes: Implications for Neoadjuvant Chemotherapy Selection, *PloS one*. 2016, *11,* e0157368.

22. Yamamoto, M.; Sakane, K.; Tominaga, K., et al. Intratumoral bidirectional transitions between epithelial and mesenchymal cells in triple-negative breast cancer, *Cancer Sci*. 2017, *108,* 1210-1222.

23. Ouelaa-Benslama, R.; De Wever, O.; Hendrix, A., et al. Identification of a GalphaGbetagamma, AKT and PKCalpha signalome associated with invasive growth in two genetic models of human breast cancer cell epithelial-to-mesenchymal transition, *International journal of oncology*. 2012, *41,* 189-200.

24. Come, C.; Magnino, F.; Bibeau, F., et al. Snail and slug play distinct roles during breast carcinoma progression, *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2006, *12,* 5395-5402.

25. Cano, A.; Perez-Moreno, M. A.; Rodrigo, I., et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression, *Nat Cell Biol*. 2000, *2,* 76-83.

26. Pattabiraman, D. R.; Weinberg, R. A. Targeting the Epithelial-to-Mesenchymal Transition: The Case for Differentiation-Based Therapy, *Cold Spring Harb Symp Quant Biol*. 2016, *81,* 11-19.

27. Mah, V.; Marquez, D.; Alavi, M., et al. Expression levels of estrogen receptor beta in conjunction with aromatase predict survival in non-small cell lung cancer, *Lung cancer*. 2011, *74,* 318-325.

28. Marquez-Garban, D. C.; Chen, H. W.; Fishbein, M. C.; Goodglick, L.; Pietras, R. J. Estrogen receptor signaling pathways in human non-small cell lung cancer, *Steroids*. 2007, *72,* 135-143.

29. Mah, V.; Seligson, D. B.; Li, A., et al. Aromatase expression predicts survival in women with early-stage non small cell lung cancer, *Cancer research*. 2007, *67,* 10484-10490.

30. Ooi, A. T.; Mah, V.; Nickerson, D. W., et al. Presence of a putative tumor-initiating progenitor cell population predicts poor prognosis in smokers with non-small cell lung cancer, *Cancer research*. 2010, *70,* 6639-6648.

31. Seligson, D. B.; Horvath, S.; McBrian, M. A., et al. Global levels of histone modifications predict prognosis in different cancers, *The American journal of pathology*. 2009, *174,* 1619-1628.

32. Yoon, N. K.; Maresh, E. L.; Elshimali, Y., et al. Elevated MED28 expression predicts poor outcome in women with breast cancer, *BMC cancer*. 2010, *10,* 335.

33. Foucquier, J.; Guedj, M. Analysis of drug combinations: current methodological landscape, *Pharmacol Res Perspect*. 2015, *3,* e00149.

34. Kalla Singh, S.; Tan, Q. W.; Brito, C.; De Leon, M.; Garberoglio, C.; De Leon, D. Differential insulin-like growth factor II (IGF-II) expression: A potential role for breast cancer survival disparity, *Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society*. 2010, *20,* 162-170.

35. Richardson, A. E.; Hamilton, N.; Davis, W.; Brito, C.; De Leon, D. Insulin-like growth factor-2 (IGF-2) activates estrogen receptor-alpha and -beta via the IGF-1 and the insulin receptors in breast cancer cells, *Growth factors*. 2011, *29,* 82-93.

36. Lehmann, B. D.; Bauer, J. A.; Chen, X., et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies, *The Journal of clinical investigation*. 2011, *121,* 2750-2767.

37. Ashton, J. C. Drug combination studies and their synergy quantification using the Chou-Talalay method--letter, *Cancer research*. 2015, *75,* 2400.

38. Chou, T. C.; Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors, *Adv Enzyme Regul*. 1984, *22,* 27-55.

39. Carey, L. A.; Perou, C. M.; Livasy, C. A., et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study, *JAMA*. 2006, *295,* 2492-2502.

40. Rida, P.; Ogden, A.; Ellis, I. O., et al. First international TNBC conference meeting report, *Breast cancer research and treatment*. 2018, *169,* 407-412.

41. Bonotto, M.; Gerratana, L.; Poletto, E., et al. Measures of outcome in metastatic breast cancer: insights from a real-world scenario, *Oncologist*. 2014, *19,* 608-615.

42. Bianchini, G.; Balko, J. M.; Mayer, I. A.; Sanders, M. E.; Gianni, L. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease, *Nat Rev Clin Oncol*. 2016, *13,* 674-690.

43. Phillips, S.; Kuperwasser, C. SLUG: Critical regulator of epithelial cell identity in breast development and cancer, *Cell Adh Migr*. 2014, *8,* 578-587.

44. Grogg, A.; Trippel, M.; Pfaltz, K., et al. Androgen receptor status is highly conserved during tumor progression of breast cancer, *BMC cancer*. 2015, *15,* 872.

45. Litzenburger, B. C.; Creighton, C. J.; Tsimelzon, A., et al. High IGF-IR activity in triple-negative breast cancer cell lines and tumorgrafts correlates with sensitivity to anti-IGF-IR therapy, *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2011, *17,* 2314-2327.

46. Davison, Z.; de Blacquiere, G. E.; Westley, B. R.; May, F. E. Insulin-like growth factor-dependent proliferation and survival of triple-negative breast cancer cells: implications for therapy, *Neoplasia*. 2011, *13,* 504-515.

47. Chang, W. W.; Lin, R. J.; Yu, J., et al. The expression and significance of insulin-like growth factor-1 receptor and its pathway on breast cancer stem/progenitors, *Breast cancer research : BCR*. 2013, *15,* R39.

48. Chakraborty, A.; Hatzis, C.; DiGiovanna, M. P. Co-targeting the HER and IGF/insulin receptor axis in breast cancer, with triple targeting with endocrine therapy for hormone-sensitive disease, *Breast cancer research and treatment*. 2017, *163,* 37-50.

49. Cassinelli, G.; Dal Bo, L.; Favini, E., et al. Supersulfated low-molecular weight heparin synergizes with IGF1R/IR inhibitor to suppress synovial sarcoma growth and metastases, *Cancer letters*. 2018, *415,* 187-197.

50. Wu, W.; Ma, J.; Shao, N., et al. Co-Targeting IGF-1R and Autophagy Enhances the Effects of Cell Growth Suppression and Apoptosis Induced by the IGF-1R Inhibitor NVP-AEW541 in Triple-Negative Breast Cancer Cells, *PloS one*. 2017, *12,* e0169229.

51. May, C. D.; Landers, S. M.; Bolshakov, S., et al. Co-targeting PI3K, mTOR, and IGF1R with small molecule inhibitors for treating undifferentiated pleomorphic sarcoma, *Cancer biology & therapy*. 2017, *18,* 816-826.

52. Hou, X.; Huang, F.; Macedo, L. F., et al. Dual IGF-1R/InsR inhibitor BMS-754807 synergizes with hormonal agents in treatment of estrogen-dependent breast cancer, *Cancer research*. 2011, *71,* 7597-7607.

53. Kim, H. J.; Litzenburger, B. C.; Cui, X., et al. Constitutively active type I insulin-like growth factor receptor causes transformation and xenograft growth of immortalized mammary epithelial cells and is accompanied by an epithelial-to-mesenchymal transition mediated by NF-kappaB and snail, *Mol Cell Biol*. 2007, *27,* 3165-3175.

54. Cevenini, A.; Orru, S.; Mancini, A.; Alfieri, A.; Buono, P.; Imperlini, E. Molecular Signatures of the Insulin-like Growth Factor 1-mediated Epithelial-Mesenchymal Transition in Breast, Lung and Gastric Cancers, *Int J Mol Sci*. 2018, *19*.

55. Mani, S. A.; Guo, W.; Liao, M. J., et al. The epithelial-mesenchymal transition generates cells with properties of stem cells, *Cell*. 2008, *133,* 704-715.

56. Wahl, G. M.; Spike, B. T. Cell state plasticity, stem cells, EMT, and the generation of intra-tumoral heterogeneity, *NPJ Breast Cancer*. 2017, *3,* 14.

57. Mallini, P.; Lennard, T.; Kirby, J.; Meeson, A. Epithelial-to-mesenchymal transition: what is the impact on breast cancer stem cells and drug resistance, *Cancer Treat Rev*. 2014, *40,* 341-348.

58. Ferrari-Amorotti, G.; Chiodoni, C.; Shen, F., et al. Suppression of invasion and metastasis of triple-negative breast cancer lines by pharmacological or genetic inhibition of slug activity, *Neoplasia*. 2014, *16,* 1047-1058.

59. Batlle, E.; Sancho, E.; Franci, C., et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells, *Nat Cell Biol*. 2000, *2,* 84-89.

60. Tripathi, M. K.; Misra, S.; Khedkar, S. V., et al. Regulation of BRCA2 gene expression by the SLUG repressor protein in human breast cells, *The Journal of biological chemistry*. 2005, *280,* 17163-17171.

61. Arteaga, C. L. Interference of the IGF system as a strategy to inhibit breast cancer growth, *Breast cancer research and treatment*. 1992, *22,* 101-106.

62. Singh, P.; Alex, J. M.; Bast, F. Insulin receptor (IR) and insulin-like growth factor receptor 1 (IGF-1R) signaling systems: novel treatment strategies for cancer, *Med Oncol*. 2014, *31,* 805.

63. Singh, B.; Smith, J. A.; Axelrod, D. M., et al. Insulin-like growth factor-I inhibition with pasireotide decreases cell proliferation and increases apoptosis in pre-malignant lesions of the breast: a phase 1 proof of principle trial, *Breast cancer research : BCR*. 2014, *16,* 463.

64. Ayub, A.; Yip, W. K.; Seow, H. F. Dual treatments targeting IGF-1R, PI3K, mTORC or MEK synergize to inhibit cell growth, induce apoptosis, and arrest cell cycle at G1 phase in MDA-MB-231 cell line, *Biomed Pharmacother*. 2015, *75,* 40-50.

65. Pollak, M. The insulin and insulin-like growth factor receptor family in neoplasia: an update, *Nature reviews Cancer*. 2012, *12,* 159-169.

66. Pollak, M. The insulin receptor/insulin-like growth factor receptor family as a therapeutic target in oncology, *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2012, *18,* 40-50.

67. Ellis, M. J.; Jenkins, S.; Hanfelt, J., et al. Insulin-like growth factors in human breast cancer, *Breast cancer research and treatment*. 1998, *52,* 175-184.

68. Rowzee, A. M.; Lazzarino, D. A.; Rota, L.; Sun, Z.; Wood, T. L. IGF ligand and receptor regulation of mammary development, *Journal of mammary gland biology and neoplasia*. 2008, *13,* 361-370.

69. Rizza, P.; Barone, I.; Zito, D., et al. Estrogen receptor beta as a novel target of androgen receptor action in breast cancer cell lines, *Breast cancer research : BCR*. 2014, *16,* R21.

70. Brys, M. Androgens and androgen receptor: do they play a role in breast cancer?, *Medical science monitor : international medical journal of experimental and clinical research*. 2000, *6,* 433-438.

71. McNamara, K. M.; Moore, N. L.; Hickey, T. E.; Sasano, H.; Tilley, W. D. Complexities of androgen receptor signalling in breast cancer, *Endocrine-related cancer*. 2014, *21,* T161-181.

72. Fioretti, F. M.; Sita-Lumsden, A.; Bevan, C. L.; Brooke, G. N. Revising the role of the androgen receptor in breast cancer, *Journal of molecular endocrinology*. 2014, *52,* R257-265.

73. Chia, K.; O'Brien, M.; Brown, M.; Lim, E. Targeting the androgen receptor in breast cancer, *Curr Oncol Rep*. 2015, *17,* 4.

74. Zhang, L.; Fang, C.; Xu, X.; Li, A.; Cai, Q.; Long, X. Androgen receptor, EGFR, and BRCA1 as biomarkers in triple-negative breast cancer: a meta-analysis, *BioMed research international*. 2015, *2015,* 357485.

75. Rakha, E. A.; El-Sayed, M. E.; Green, A. R.; Lee, A. H.; Robertson, J. F.; Ellis, I. O. Prognostic markers in triple-negative breast cancer, *Cancer*. 2007, *109,* 25-32.

76. Tang, D.; Xu, S.; Zhang, Q.; Zhao, W. The expression and clinical significance of the androgen receptor and E-cadherin in triple-negative breast cancer, *Med Oncol*. 2012, *29,* 526-533.

77. Giannos, A.; Filipits, M.; Zagouri, F., et al. Expression of ARs in triple negative breast cancer tumors: a potential prognostic factor?, *Onco Targets Ther*. 2015, *8,* 1843-1847.

78. Hu, D. G.; Hickey, T. E.; Irvine, C., et al. Identification of androgen receptor splice variant transcripts in breast cancer cell lines and human tissues, *Hormones & cancer*. 2014, *5,* 61-71.

79. Mehrian-Shai, R.; Chen, C. D.; Shi, T., et al. Insulin growth factor-binding protein 2 is a candidate biomarker for PTEN status and PI3K/Akt pathway activation in glioblastoma and prostate cancer, *Proceedings of the National Academy of Sciences of the United States of America*. 2007, *104,* 5563-5568.

80. Gonzalez-Angulo, A. M.; Stemke-Hale, K.; Palla, S. L., et al. Androgen receptor levels and association with PIK3CA mutations and prognosis in breast cancer, *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2009, *15,* 2472-2478.

81. KM, C.; J, L.; GD, F.; A, N. A feedback loop between androgen receptor and ERK signaling in estrogen receptor-negative breast cancer, *Neoplasia*. 2011, *13,* 154-166.

82. Zhu, M. L.; Kyprianou, N. Androgen receptor and growth factor signaling cross-talk in prostate cancer cells, *Endocrine-related cancer*. 2008, *15,* 841-849.

83. Zhu, A.; Li, Y.; Song, W., et al. Antiproliferative Effect of Androgen Receptor Inhibition in Mesenchymal Stem-Like Triple-Negative Breast Cancer, *Cell Physiol Biochem*. 2016, *38,* 1003-1014.

84. Liu, C. Y.; Lau, K. Y.; Hsu, C. C., et al. Combination of palbociclib with enzalutamide shows in vitro activity in RB proficient and androgen receptor positive triple negative breast cancer cells, *PloS one*. 2017, *12,* e0189007.

85. Xu, L.; Qi, Y.; Xu, Y., et al. Co-inhibition of EGFR and IGF1R synergistically impacts therapeutically on adrenocortical carcinoma, *Oncotarget*. 2016, *7,* 36235-36246.

86. Bielen, A.; Perryman, L.; Box, G. M., et al. Enhanced efficacy of IGF1R inhibition in pediatric glioblastoma by combinatorial targeting of PDGFRalpha/beta, *Molecular cancer therapeutics*. 2011, *10,* 1407-1418.

87. Lin, H. K.; Wang, L.; Hu, Y. C.; Altuwaijri, S.; Chang, C. Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase, *EMBO J*. 2002, *21,* 4037-4048.

88. Yahya, M. A.; Sharon, S. M.; Hantisteanu, S.; Hallak, M.; Bruchim, I. The Role of the Insulin-Like Growth Factor 1 Pathway in Immune Tumor Microenvironment and Its Clinical Ramifications in Gynecologic Malignancies, *Frontiers in endocrinology*. 2018, *9,* 297.

89. Emens, L. A.; Cruz, C.; Eder, J. P., et al. Long-term Clinical Outcomes and Biomarker Analyses of Atezolizumab Therapy for Patients With Metastatic Triple-Negative Breast Cancer: A Phase 1 Study, *JAMA Oncol*. 2019, *5,* 74-82.