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Anastrozole has an association between degree of estrogen suppression and outcomes in early breast cancer and is a ligand for estrogen receptor α

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Abstract

Purpose—To determine if the degree of estrogen suppression with aromatase inhibitors (AIs: anastrozole, exemestane, letrozole) is associated with efficacy in early stage breast cancer, and to examine for differences in the mechanism of action between the three AIs.

Experimental Design—Matched case-control studies [247 matched sets from MA.27 (anastrozole vs. exemestane) and PreFace (letrozole) trials] were undertaken to assess whether estrone or estradiol concentrations after six months of adjuvant therapy were associated with risk of an early breast cancer event (EBCE). Preclinical laboratory studies included luciferase activity, cell proliferation, radio-labeled ligand estrogen receptor binding, surface plasmon resonance ligand receptor binding, and nuclear magnetic resonance assays.

Results—Women with estrone   1.3 pg/mL and estradiol   0.5 pg/mL after six months of AI treatment had a 2.2-fold increase in risk (p=0.0005) of an EBCE, and in the anastrozole subgroup, the increase in risk of an EBCE was 3.0-fold (p=0.001). Preclinical laboratory studies examined mechanisms of action in addition to aromatase inhibition and showed that only anastrozole could directly bind to ERα, activate estrogen response element-dependent transcription, and stimulate growth of an aromatase-deficient *CYP19A1*$^{-/-}$ T47D breast cancer cell line.

Conclusions—This matched case-control clinical study revealed that levels of estrone and estradiol above identified thresholds after six months of adjuvant anastrozole treatment were associated with increased risk of an EBCE. Preclinical laboratory studies revealed that anastrozole, but not exemestane or letrozole, is a ligand for estrogen receptor α. These findings represent potential steps towards individualized anastrozole therapy.

Keywords

aromatase inhibitors; anastrozole; exemestane; letrozole; breast cancer; estrogen suppression; estrogen receptor α

INTRODUCTION

The third-generation aromatase inhibitors (AIs) anastrozole, exemestane and letrozole play a major role in the adjuvant therapy of postmenopausal women with early-stage estrogen receptor α (ERα)-positive breast cancer (1,2). The assumption is that the mechanism of action of all three AIs in inhibiting tumor growth is only through decreasing estrogen production from androgenic precursors by inhibiting aromatase (3), which is encoded by the *CYP19A1* gene (4–6). Whereas all three AIs are efficacious in terms of inhibition of *in vivo* aromatization (7–9), letrozole has been found to be the most potent (10). However, large phase III adjuvant clinical trials do not indicate any difference in efficacy between the three AIs. Specifically, the MA.27 trial showed no significant difference between anastrozole and exemestane (11), and the FACE trial showed no difference between anastrozole and letrozole (12). It has, however, been reported that the relative efficacy, *vis-à-vis* tamoxifen, of anastrozole (13), but not exemestane (14) or letrozole (15), is worse in obese patients who have increased concentrations of estrogens (16).

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We previously performed a pharmacokinetic and pharmacodynamic study in 649 patients receiving anastrozole adjuvant therapy (17) that showed 30% and 21% had a broad range of detectable estrone (E1) and estradiol (E2) concentrations, respectively, above the lower limit of quantitation (LLQ) after at least four weeks of therapy. No clinical studies have systematically tested whether the degree of estrogen suppression after achieving steady state concentrations of an AI is associated with AI efficacy in early breast cancer. To address this question, we performed a matched case-control study that examined the association between risk of an early (within five years of starting AI therapy) breast cancer event (EBCE) and estrogen suppression among women treated with anastrozole, exemestane, or letrozole, and found concentrations of E1 and E2 after six months of AI treatment that were associated with a significantly increased risk of an EBCE. Examination of this association within each treatment group was limited due to small sample sizes but this association was found to be significant for those treated with anastrozole. Preclinical laboratory studies were performed to determine if there were any mechanisms of action, in addition to inhibition of aromatase, for the three AIs and these revealed that anastrozole, but not exemestane or letrozole, functions as an ERα ligand. In total, these findings may have profound implications for patient management when patients are considered for anastrozole therapy.

METHODS

Source of Patients

MA.27—The MA.27 phase III trial (ClinicalTrials.gov identifier NCT00066573) was conducted by the North American Breast Intergroup and included postmenopausal (criteria in Supplementary Material) women with resected stage I-III breast cancer (AJCC Version 6) who were randomized to anastrozole (1 mg/day) or exemestane (25 mg/day) as adjuvant therapy with a planned treatment duration of five years. Only North American patients [6827 of 7576 (90%) of MA.27 accrual] were offered participation in collection of blood specimens and 5221 (76.5%) of the North American patients contributed blood and gave consent for genetic testing. Plasma samples were obtained at baseline and at six months in an EDTA-containing tube and patients were to be instructed to avoid alcohol for 48 hours and fast for 14 hours before the blood draw.

PreFace—PreFace (Evaluation of **Pre**dictive **Fac**tors for the **E**ffectivity of Aromatase Inhibitor Therapy, ClinicalTrials.gov identifier NCT01908556) was a prospective open-labeled multicenter phase IV trial of letrozole at a dose of 2.5 mg/day with a planned treatment duration of five years in postmenopausal (criteria in Supplementary Material) women with early stage breast cancer that recruited 3475 patients between December 2008 and August 2010. The goal of this trial was to examine the influence of biomarkers that could predict the efficacy and side effects of adjuvant letrozole therapy. The trial was sponsored by the Institut fur Frauengesundheit, GmbH, Erlangen, Germany, academically led by the commission for translational research of the working group for Gynecologic Oncology [Arbeitsgemeinschaft für Gynäkologische Onkologie (AGO)] and conducted in 220 breast centers in Germany. All patients had a blood sample obtained for DNA and serum samples pretreatment and at six and 12 months.

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This research was performed after approval by local institutional review boards in accordance with assurances filed with, and approved by, the Department of Health and Human Services.

Design of Matched Case-Control Studies—A nested matched case-control approach (18) was used where cases were women who developed an EBCE within five years of starting AI therapy and controls were those with disease-free follow-up at least six months longer than the case, were within five years of age, had the same disease stage (stage I, II, or III), same body mass index (BMI) category (<25.0, 25.0–34.9, or >35), and same adjuvant chemotherapy status (yes or no) as the case. Specifically excluded were patients with bilateral breast cancers, breast primaries that were Tx or T4, age >85 years, second non-breast primary prior to breast event, and non-detectable AI concentrations at six months. For MA.27, an attempt was made to identify two controls for each case (19,20). For the PreFace study, an attempt was made to identify up to three controls for each case when we found that the number of cases was less than anticipated.

An EBCE was considered to be any of the following: local-regional breast cancer recurrence [including ipsilateral DCIS], distant breast cancer recurrence, contralateral breast cancer [invasive or DCIS] or death with or from breast cancer without prior recurrence.

Estrone and estradiol Assays—Pre- and post-AI treatment E1 and E2 levels were measured by CLIA-approved liquid chromatography with tandem mass spectrometry (LC/MS/MS) assays in the Immunochemical Core Laboratory at Mayo Clinic. Details of the methodology have been published (21–23) and additional information is given in the Supplementary Material. Intra-assay CVs for E1 are 17.8%, 7.5%, and 6.1% at 0.30, 0.50, and 0.84 pg/mL, respectively. Intra-assay coefficients of variation (CV) for E2 are 11.8%, 7.3%, and 6.0%, at 0.23, 0.50, and 0.74, pg/mL, respectively. Inter-assay CVs for E1 are 12.0%, 9.5%, and 7.9% at 0.25, 0.51, and 0.85 pg/mL, respectively. Inter-assay CVs for E2 are 10.8%, 8.5%, and 6.9% at 0.29, 0.50, and 0.77 pg/mL, respectively.

Aromatase Inhibitor Assays—Anastrozole, exemestane, and letrozole and relevant metabolites were measured in the laboratory of Zeruesenay Desta, Ph.D. at Indiana University School of Medicine using liquid chromatography-tandem mass spectrometry (LC/MS/MS) assays. Details regarding sample preparation, LC/MS/MS methodology, and calibration curve preparation are given in the Supplementary Material. The calibration standards and quality controls were judged for batch quality based on the US Food and Drug Administration guidance for industry regarding bioanalytical method validation. For anastrozole and letrozole, the LLQ was 0.07 ng/mL with intra-day and inter-day CV of 7.1% and 14.5%, respectively, and an intra-assay and inter-assay CV of <7.1% and <15%, respectively. For exemestane the LLQ was 0.071 ng/mL, with intra-day and inter-day CV of 11.2%, respectively, and an intra-assay and inter-assay CV of 11.2% and 16.8%, respectively.

Statistical Design—The original statistical analysis plan was to utilize the MA.27 cohort as the discovery set to determine whether there was a threshold for E1 and/or E2 after six months of treatment that conferred a higher risk of EBCE and then to attempt to validate

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these findings in PreFace cohort. This approach was abandoned due to lower than expected number of cases meeting eligibility criteria or having sufficient plasma or serum in the MA27 cohort (186 [74.4%] of the anticipated 250 cases) and in the PreFace cohort (61 [24.4%] of the anticipated 250 cases). Instead, the matched case-control sets constructed from MA27 and PreFace trial cohorts were analyzed together to determine whether there was a level of E1 or E2 after six months of treatment that confers a higher risk of an EBCE.

Due to the lack of an independent validation cohort, a bootstrap re-sampling approach was undertaken to determine 'best' cut points for the biomarkers, E1 and E2. Specifically, 500 bootstrap samples were constructed by sampling with replacement 222 matched case-control sets from the 247 matched case-control sets. The set of potential cut-points assessed for each biomarker included values from its lower limit of quantification to its 85th percentile value (across all women). The 'best' cut point for a given biomarker was chosen using the maximum concordance approach of Liu (24). Specifically, for each potential cut point y_i, an indicator variable x_i was constructed where x_i=1 if the patient's biomarker value was at or above y_i; x_i=0 otherwise. Then, for each bootstrap sample, j, and each cut point, i, a concordance statistic C_{ij} was generated from fitting a stratified Cox model with case-control set as the strata, time set to the constant of 1, and the indicator variable for the cut point was fit to the data (25). A stratified Cox model fit in this manner is equivalent to the conditional logistic regression model, which is appropriate for analysis of matched case-control data. The cut point where the maximum value of the concordance statistic, max C_j, occurred was determined for each bootstrap sample j. The cut point most often found to have the max C_j cut point across the 500 samples was chosen for further evaluation. Having established cut points for E1 and E2, multivariate conditional logistic regression modeling using all 247 matched pairs was used to refine the model of risk.

A secondary exploratory analysis was carried out using the refined model to obtain an estimate of the odds of an EBCE associated with E1 and E2 thresholds for each treatment cohort separately. Statistical analyses were carried out using SAS 9.4 and the survConcordance (25) function in the survival package of R software.

Spearman rank correlation coefficients were used to assess associations between E1 and E2 serum concentrations and results that fell below the LLQ were set at one-half of the LLQ value.

Laboratory Studies

Cell lines—Human ER+ breast cancer cell lines T47D and MCF7 were obtained from American Type Culture Collection (ATCC, Manassus, VA) in 2014 and the identities of all cell lines were confirmed by the medical genome facility at Mayo Clinic (Rochester MN) using short tandem repeat profiling upon receipt.

Radio-labeled ligand receptor binding assay—Recombinant full length human ERα protein was diluted in ice-cold assay buffer (10 mM Tris-HCl; 1 mM EDTA; 1 mM EGTA; 1 mM NaVO3; 1% glycerol; 0.25 mM leupeptin; 1% BSA; 1 mM DTT). [^{3}H]-Anastrozole (Moravek) and [^{3}H]-17β-estradiol (PerkinElmer) ERα saturation binding assays were performed to measure total binding (TB) and non-specific binding (NSB). To determine

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nonspecific binding, a 500-fold excess of non-radioactive ligand was incubated overnight together with 0.5 nM ERα protein and radioactive ligand at 4 °C. Bound and unbound ligands were separated by incubation with ice-cold dextran-coated charcoal (DCC) on ice for 10 min. After centrifugation for 15 mins at 8000 rpm, 100 µl of DCC suspension was gently transferred into LSC vials containing 1 mL Ultima Gold scintillation cocktail (Perkin Elmer Life Sciences, Boston, MA, USA). Radioactivity was then measured using a Beckmann LS 6500 liquid scintillation counter (Ramsey, MN, USA). Specific binding (SB) was calculated by subtracting the value for NSB from that for TB. The equilibrium dissociation constant for the radioligand (K_D) was calculated using non-linear regression analysis (GraphPad Prism Software v7, San Diego, CA, USA). [^{3}H]-Anastrozole displacement assays were performed in a similar fashion using 100 nM [^{3}H]-Anastrozole and increasing concentrations of non-radioactive ligand, i.e. non-radioactive E2 or anastrozole (0.01 nM–1000 µM). Separation of bound and unbound radioligands was performed as described above. Competition curves were plotted as the percentage of SB of radioactive ligand versus increasing concentrations of non-radioactive ligand. The radioligand binding assays were performed in three independent experiments. [^{3}H]-17β-estradiol displacement assays were performed in a similar fashion.

Surface Plasmon Resonance (SPR) Ligand Receptor Binding Assay— Nitrilotriacetic acid mixed capture of full length H_{is6}-tagged human ERα (Creative Biomart, ESRH1) was performed using a Biacore T200 SPR analyzer (GE Healthcare). Briefly, the NTA chip (BR-1000–34) was conditioned with 350 mM EDTA (pH 8.3) for 1 min followed by washing with immobilization buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.01 % polysorbate) for 5 min at a flow 30 µl/min. After a 0.5 mM $NiCl_2$ injection (60 s), the H_{is6}-tagged ERα (0.2 mg/mL) was captured at a flow of 5 µL/min. After capture, the ligand was cross-linked using amine coupling with a 1 min pulse of N-ethyl-N′-[dimethylaminopropyl]carbodiimide/N-hydroxysuccinimide and ethanolamine, and reached a level of 8000–10000 resonance units (RU). Anastrozole, letrozole, exemestane and estradiol, at concentrations from 0.025–20 µM in calcium- and magnesium-free Dulbecco’s phosphate buffered saline containing 2% DMSO and 0.01% polysorbate, were passed over the chip surface at flow rate of 50 µl/min for 30 s and allowed to dissociate for 60 s. Kinetic analysis of SPR data was performed using BiaEvaluation (GE). Sensograms were subtracted for background and DMSO contributions, and affinity constants were derived using a steady state affinity fitting 1:1 interaction model.

Additional laboratory methods—Details regarding cell culture techniques, generation of *CYP19A1* knockout cells by CRISPR/Cas9 technology, cell proliferation assays, luciferase activity assays, siRNA transfection and quantitative real-time PCR assay (qRT-PCR), Western blotting, and the Carr-Purcell-Meiboom-Gill (CPMG) NMR assay are given in the Supplementary Material.

RESULTS

The REMARK (26) diagrams for the MA.27 trial (Supplementary Fig. S1) and the PreFace trial (Supplementary Fig. S2) show the patients included in, and excluded from, the case-control studies. The analysis included 186 cases and 327 controls from MA27 and 61 cases

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and 171 controls from PreFace (Table 1). Most of the patients were white (MA.27: 94.3%, PreFace: 99.6%), and two-thirds of the cases from both MA.27 and PreFace had distant metastasis as their first disease event.

For MA.27, a comparison of the cases who did and did not have a blood sample available found that there was balance (no significant difference) for the categories of age, BMI, and lymph node status, but cases who did have a blood sample were more likely to have higher stage disease and receive prior adjuvant chemotherapy than those who did not. PreFace cases with a blood sample did not differ significantly from cases without a blood sample for any of the variables age, BMI, stage, lymph node status, or prior adjuvant chemotherapy, although there was a trend for cases with a blood sample to be younger (p=0.08) (Supplementary Table S1).

Estrone, Estradiol, and AI Concentrations

The pre-AI E1 and E2 concentrations were similar among the three treatment cohorts and were highly correlated within each treatment cohort with Spearman rank coefficients (ρ) of 0.84 for anastrozole, 0.89 for exemestane, and 0.90 for letrozole (Supplementary Fig. S3). After six months of AI therapy, E1 and E2 concentrations were below the LLQ in 41.3% and 11.9% of the patients, respectively, for anastrozole; 63.7% and 35.4%, respectively, for exemestane; and 79.3 % and 48.7%, respectively, for letrozole (Table 2). The correlation between E1 and E2 concentrations after six months of AI therapy was moderate for anastrozole (ρ= 0.54) and exemestane (ρ=0.52) but weak for letrozole (ρ= 0.15) (Supplementary Fig. S4). There was a very weak correlation between both E1 and E2 with AI concentrations after 6 months of treatment with ρ= −0.20 and −0.12 for anastrozole, −0.18 and −0.12 for exemestane, and −0.11 and −0.09 for letrozole, respectively (Supplementary Fig. S5). Six-month concentrations of each of the AIs showed substantial inter-patient variability (Table 2, Supplementary Fig. S6 A–C). After six months on AI therapy, most patients had a decrease in both E1 and E2 although a small percentage of patients had an increase in E1 (0.4%–1.7%) and/or E2 (2.1%–5.2%) (Table 2, Supplementary Fig. S7 A–C).

Estrone and Estradiol Suppression and Risk of an Early Breast Cancer Event

We examined cut points and their association with EBCEs for E1 after six months of AI treatment between its lower limit of quantification (1.0 pg/mL) and the 85th percentile value of its distribution (1.7 pg/mL). We utilized a bootstrap re-sampling approach where each of the 500 bootstrap samples was constructed by sampling, with replacement, 222 of the 247 matched case-control sets. For each bootstrap sample and each cut point, conditional logistic regression was performed and an estimate of the odds of an EBCE and the concordance statistic were determined. From the 500 estimates of the odds ratio for a given cut point and the median and 2.5^{th} and 97.5^{th} percentile value of its distribution were determined (Figs. 1A). The number of times a given cut point for E1 was associated with the maximum concordance value among the 500 bootstrap samples was also calculated (Figs. 1C). The value of E1 most often having the maximum concordance value was 1.3 pg/mL (Fig. 1C). Assessed in 245 matched case-control sets with E1 data available (2/247 sets had missing E1 data), women with an E1   1.3 pg/mL after six months of AI therapy had a 1.75 fold

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(95%CI: 1.19–2.55, p=0.004) increased risk of an EBCE relative to those with an E1 < 1.3 pg/mL.

The process was repeated for E2 searching between the lower limit of quantification (0.3 pg/mL) and 85th percentile value of its pre-treatment distribution (0.7 pg/mL) (Fig. 1B). The number of times a given cut point was associated with the maximum concordance value among the 500 bootstrap samples was also calculated (Fig. 1D). The value of E2 most often having the maximum concordance value among the 500 bootstrap samples was 0.5 pg/mL. Utilizing the entire dataset of 247 matched case-control sets, women with an E2 0.5 pg/mL after six months of AI therapy had a 1.44 fold (95%CI: 1.02–2.03, p=0.04) increased risk of an EBCE relative to those with an E2< 0.5 pg/mL.

In multivariate analysis assessing both E1 and E2 together, conditional logistic regression found that women only one of either E1 1.3 pg/mL or E2 0.5 pg/mL did not differ significantly from women with both E1<1.3 and E2<0.5 (odds ratio 1.05, 95% CI: 0.69–1.58, p=0.83) (Supplementary Table 2). Thus, our final model collapsed these two categories and only included the effect for both E1 1.3 pg/mL and E2 0.5 pg/mL versus not. Women with both E1 1.3 pg/mL and E2 0.5 pg/mL had a 2.2-fold (95% CI: 1–42-3.47, p=0.0005) increase in risk of an EBCE relative to women with E1 and/or E2 below these thresholds after six months of AI treatment.

The question as to whether the relationship between having both E1 and E2 above their respectively thresholds (i.e., E1 1.3 pg/mL and E2 0.5 pg/mL) after six months of AI treatment and risk holds for each treatment group was carried out in an exploratory manner given the small sample sizes. We found that the risk of an EBCE was increased 3.0-fold (matched case-control sets=91: 95% CI: 1.56–5.76, p=0.001) for those with E1 and E2 values at or above their threshold after six months of anastrozole therapy. However, the result was not significant in patients treated with exemestane (matched case-control sets=95: 95% OR=1.66; CI: 0.82–3.33, p=0.16) or letrozole (matched case-control sets=59: 95% OR=1.62; CI: 0.39–6.82, p=0.51) (Fig. 1E).

Laboratory Studies

We performed a series of laboratory studies to examine for any differences in the mechanisms of action of anastrozole, exemestane and letrozole.

Anastrozole Differs from Exemestane and Letrozole with Regard to Growth of *CYP19A1* CRISPR Knock Out (KO) T47D Cells

To eliminate the known mechanism of aromatase inhibition by AIs, we first created *CYP19A1* CRISPR KO T47D cells to determine whether there might be an additional mechanism of action for anastrozole. Under estrogen-free conditions, the CYP19A1 KO T47D cells proliferated slower than wild type (WT) T47D cells under the same culture conditions (Fig. 2A). Compared with vehicle treatment, all three AIs could slow the proliferation of WT T47D cells that contained the aromatase enzyme in a dose-dependent fashion (Supplementary Fig. S8A). However, in *CYP19A1* KO T47D cells, only anastrozole at 0.1–10 nM resulted in increased cell proliferation, with a decrease in cell proliferation when anastrozole concentrations increased to 100–500 nM (Fig. 2B). In *CYP19A1* KO

T47D cells, treatment with exemestane or letrozole at the same concentrations (0.1 to 500 nM) did not affect cell proliferation compared with vehicle-treated cells (Supplementary Fig. S8B).

Anastrozole Acts as an ERα Ligand

Previous studies showed that CYP17 inhibitor, abiraterone could bind to the androgen receptor (27). Therefore, we hypothesized that anastrozole might function through ERα. We tested the three AIs for their ability to inhibit E2-stimulated ERα activity in *CYP19A1* CRISPR KO T47D cells as well as parental T47D, and MCF7 cells using an estrogen response element (ERE)-luciferase reporter assay as a readout for ERα activity. In all three cell lines, we observed a striking dose-dependent increase in luciferase activity with anastrozole, similar to the effect of E2 (Fig. 2C, Supplementary Fig. S8C). However, anastrozole failed to induce luciferase activity in ESR1 knockdown cells (Supplementary Fig. S8D). These observations indicated that anastrozole behaves similarly to E2 with regard to its effect on ERE-dependent transcription activation.

To determine whether anastrozole directly interacts with ERα, we employed three methodologies. Radioligand binding assays using [^{3}H]-anastrozole revealed a KD = 185.1 ± 21.66 nM based on non-linear regression analysis of the saturation binding assay (Fig. 2D, upper panel). To determine whether anastrozole binds to the same site on ERα as E2, radio-label competition binding assays were performed at a fixed concentration of [^{3}H]-anastrozole in the presence of increasing concentrations of non-radioactive E2 or anastrozole. Non-radioactive anastrozole competed with [^{3}H]-anastrozole for binding to ERα, but E2 was not able to compete off the binding of [^{3}H]-anastrozole to ERα (Fig. 2D, lower panel). Similarly, competition binding assays using a fixed concentration of [^{3}H]-E2 showed that anastrozole, as well as exemestane and letrozole, did not compete off the binding of [^{3}H]-E2 to ERα (Supplementary Fig. S8E), indicating that anastrozole and E2 bind to different sites on ERα. The anastrozole-ERα binding was further confirmed using two biophysical techniques. First, surface plasmon resonance spectroscopy(28) demonstrated that anastrozole bound to ERα protein (Fig. 2E, upper panel) whereas binding of exemestane and letrozole could not be detected (Fig. 2E lower panel). Second, using Carr-Purcel- Meiboom-Gill NMR spectroscopy(29), we monitored the one-dimensional proton (^{1}H) NMR signal of anastrozole in the presence and absence of the ERα protein to determine if a non-covalent binding interaction was present. A decrease in the ^{1}H NMR signals for anastrozole was observed for anastrozole in the presence of ERα, consistent with binding to the receptor (Fig. 2F). Our data suggested that anastrozole behaved in a fashion similar to E2 in that it can activate ERα-dependent transcription, but the effect decreased with increasing concentrations of anastrozole (Fig. 2C, Supplementary Fig. S8C).

Given that E2 can induce ERα degradation (30) we tested whether anastrozole might also influence ERα protein degradation. Treatment with 100 nM anastrozole decreased ERα protein level in *CYP19A1* CRISPR KO T47D cells, while exemestane or letrozole at the same concentration did not alter ERα protein levels (Fig. 2G, left panel). Note that the median anastrozole concentration in the case-control study was 27.2 ng/mL, which is about

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92.7 nM. In the presence of 100 nM anastrozole, we also observed a time-dependent ERα protein degradation (Fig. 2G, right panel).

We then tested the effect of anastrozole on cell proliferation in the presence of estrogen levels below, at, and above the thresholds identified in the clinical studies described above. We converted the *in vivo* plasma concentrations to *in vitro* levels. Treating cells at the concentrations that equivalent to plasma E1 and E2 concentrations that were below 1.3 pg/mL and 0.5 pg/mL threshold, respectively, had little effect on *CYP19A1* CRISPR KO T47D cell proliferation (Supplementary Fig. S9A). Anastrozole, but not exemestane or letrozole, potentiated estrogen effects on cell proliferation when estrogen levels were above the thresholds, compared to estrogen alone (Supplementary Figs. S9A, S10), especially when both E1 and E2 were above the thresholds (Fig. 3 A–C). Anastrozole, but not exemestane or letrozole, potentiated estrogen-induced ERE luciferase activity when estrogen levels were above the thresholds (Fig. 3 D–F, Supplementary Fig. S9B). These phenomena were also observed in an MCF7 cell line in which anastrozole, but not exemestane or letrozole potentiated estrogen effects on cell proliferation when estrogen levels were above the thresholds, compared to estrogen alone (Supplementary Figs. S11A, S12), especially when both E1 and E2 were above the thresholds (Fig. 4 A–C). Anastrozole, but not exemestane or letrozole, also potentiated estrogen-induced ERE luciferase activity when estrogen levels were above the thresholds (Fig. 4 D–F, Supplementary Fig. S11B).

DISCUSSION

Our matched case-control study of postmenopausal women with resected early stage breast cancer treated with anastrozole, exemestane or letrozole adjuvant therapy revealed that a woman who, after six months of AI therapy had an E1   1.3 pg/mL and an E2   0.5 pg/mL had a statistically significant (p=0.0005) and clinically meaningful 2.2-fold increased risk of an EBCE compared to a woman with the same matching characteristics (age within five years, stage, BMI category, and presence or absence of prior chemotherapy) but who had an E1 and/or E2 below these respective thresholds. Based on our matched case-control studies, E1 and E2 appear to be biomarkers associated with outcomes in postmenopausal women treated with AIs.

We utilized a bootstrap resampling approach for an internal validation. Ideally, we would have had a sufficiently large population of patients to perform a discovery study followed by a validation study. Given our findings, it would also have been ideal to have large populations of patients receiving each of the AIs, given the unexpected findings of a difference between anastrozole and the other two AIs, exemestane and letrozole. These represent limitations of our study. Unfortunately, no other large adjuvant trial of monotherapy with these AIs collected serum or plasma that would have made pharmacodynamic studies possible.

The position that the three third-generation AIs can be considered to have a “class effect” based on a similar mechanism of action (i.e., inhibition of aromatase) and the finding of similar efficacy for anastrozole and exemestane in the MA.27 trial (11) and anastrozole and letrozole in the PreFace trial (12) is challenged by the results reported here. In analyzing the

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results for each AI, we confirmed previous reports that letrozole is the most potent of the three AIs as shown by the higher percentage of patients having six-month E1 and E2 concentrations below the LLQ (Table 2). Individually, however, only anastrozole showed a significant association between the six-month E1 and E2 concentrations and risk of an EBCE. That is, for women with E1 and E2 concentrations at or above their respective threshold after six months of anastrozole, the risk of an EBCE was increased 3.0-fold (95% CI: 1.56–5.76, p=0.001). However, we recognize the limitations of the analyses for the individual AIs because of small sample sizes and additional adequately powered studies are needed to clarify the role of E1 and E2 suppression and the risk of EBCE in these treatment groups.

Support for the position that anastrozole differs from exemestane and letrozole is provided by our preclinical laboratory studies, which show that only anastrozole stimulated cell proliferation in breast cancer cell lines (Fig. 2B, Supplementary Fig. S8B). The studies in T47D cells with *CYP19A1* knock out removed the known target of AIs and indicated that anastrozole has a mechanism of action in addition to inhibition of aromatase. Anastrozole, but neither exemestane nor letrozole, was then shown to activate ERE-dependent transcription in not only WT T47D and MCF7 cells, but also in *CYP19A1* knock out T47D cells (Fig. 2C, Supplementary Fig. S8C). We then performed radioligand binding assays (Fig. 2D), surface plasmon resonance (Fig. 2E), and NMR studies (Fig. 2F) that confirmed anastrozole binds to ERα. Additional dose-response ERE luciferase studies showed a bell-shaped curve for anastrozole, similar to E2, with lower doses being stimulatory and higher doses being inhibitory and associated with degradation of ERα (Figs. 2C, 2G).

A major question is the mechanism by which patients with E1 and E2 levels at or above their respective thresholds (1.3 and 0.5 pg/mL, respectively) have an increased risk of an EBCE when treated with anastrozole. Based on our preclinical data, in which we attempted to mimic the E1, E2, and AI concentrations seen clinically, we hypothesize that in these cases the ERα agonistic effect of anastrozole might combine with the agonistic effect of E1 and E2 to stimulate the proliferation of micrometastasis as the results demonstrated in *CYP19A1* knock out T47D cells as well as wild type MCF7 cells (Figs. 3A, 4A). Future mechanistic studies of anastrozole-dependent ERα regulation need to be performed.

The findings from our studies raise the possibility of salvaging anastrozole-treated patients identified to be at the three-fold increased risk of an EBCE. Whereas patients could be switched to an alternative AI, an additional approach would be to treat these patients with a higher dose of anastrozole in order to take advantage of the ability of anastrozole to degrade ERα at higher doses. There is extensive clinical experience with anastrozole at 10 mg per day, which, in two prospective randomized trials (31), was shown overall to be equal to a 1 mg dose in terms of efficacy and tolerability in the metastatic breast cancer setting. The ideal setting to study this approach would be in the neoadjuvant setting where the effects of increasing the dose of anastrozole on pharmacodynamics, proliferation, and ERα could be studied.

In summary, we have shown for the first time that higher levels, i.e. at or above specific thresholds, of E1 and E2 after six months of treatment are associated with significantly

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higher risk of an EBCE when treated with anastrozole at the standard dose of 1 mg/day. Mechanistically, we have shown that anastrozole has a mechanism of action in addition to aromatase inhibition in that it is a ligand for ERα. These findings have implications for individualization of anastrozole therapy because the identification of patients at high risk of an EBCE might be salvaged by use of an alternative therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Conflict of interest statement: P.A.F. has received honoraria from Novartis, Pfizer, Roche, Amgen, Celgene, Daiichi-Sankyo, AstraZeneca, Merck-Sharp & Dohme, Eisai, Puma, and Teva. His institution conducts research with funding from Novartis and BioNTech. M.J.E. reports employment with Bioclassifier, LLC; royalty income from Prosigma/Nanostring; honoraria from Nanostring, Novartis, AstraZeneca, Pfizer, Sermonix, Abbvie; patent interest from Bioclassifier, LLC, Prosigma/PAM50; P.E.G. reports research funding from Amgen; M.P.G. reports consulting roles with Lilly, Bovica, Novartis, Sermonix, Context Pharmaceuticals, and Genomic Health; S.H.K reports intellectual property interest with Millipre-Sigma and Topagen; R.M.W. and L.W. are co-founders of, and stockholders in OneOme, LLC. All other authors have declared that they have no potential conflicts of interest

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STATEMENT OF TRANSLATIONAL RELEVANCE

Aromatase inhibitors (AIs) play an integral role in the adjuvant therapy of early stage estrogen receptor α (ERα)-positive breast cancer and current dogma considers the three AIs anastrozole, exemestane, and letrozole as interchangeable clinically. Our case-control study revealed that levels of estrone (E1) and estradiol (E2) at or above specific thresholds (E1   1.3 pg/mL, E2   0.5 pg/mL) after six months of adjuvant AI therapy were associated with increased risk of an early breast cancer event (EBCE). Analysis according to AI revealed anastrozole to have a significant three-fold risk of an EBCE when E1 and E2 were at or above their respective thresholds. In preclinical laboratory studies, anastrozole, but not exemestane or letrozole, was a ligand for ERα thus having a mechanism of action in addition to inhibition of aromatase. These findings represent potential steps towards individualized anastrozole therapy and provide potential for exploiting anastrozole's action as an ERα ligand.

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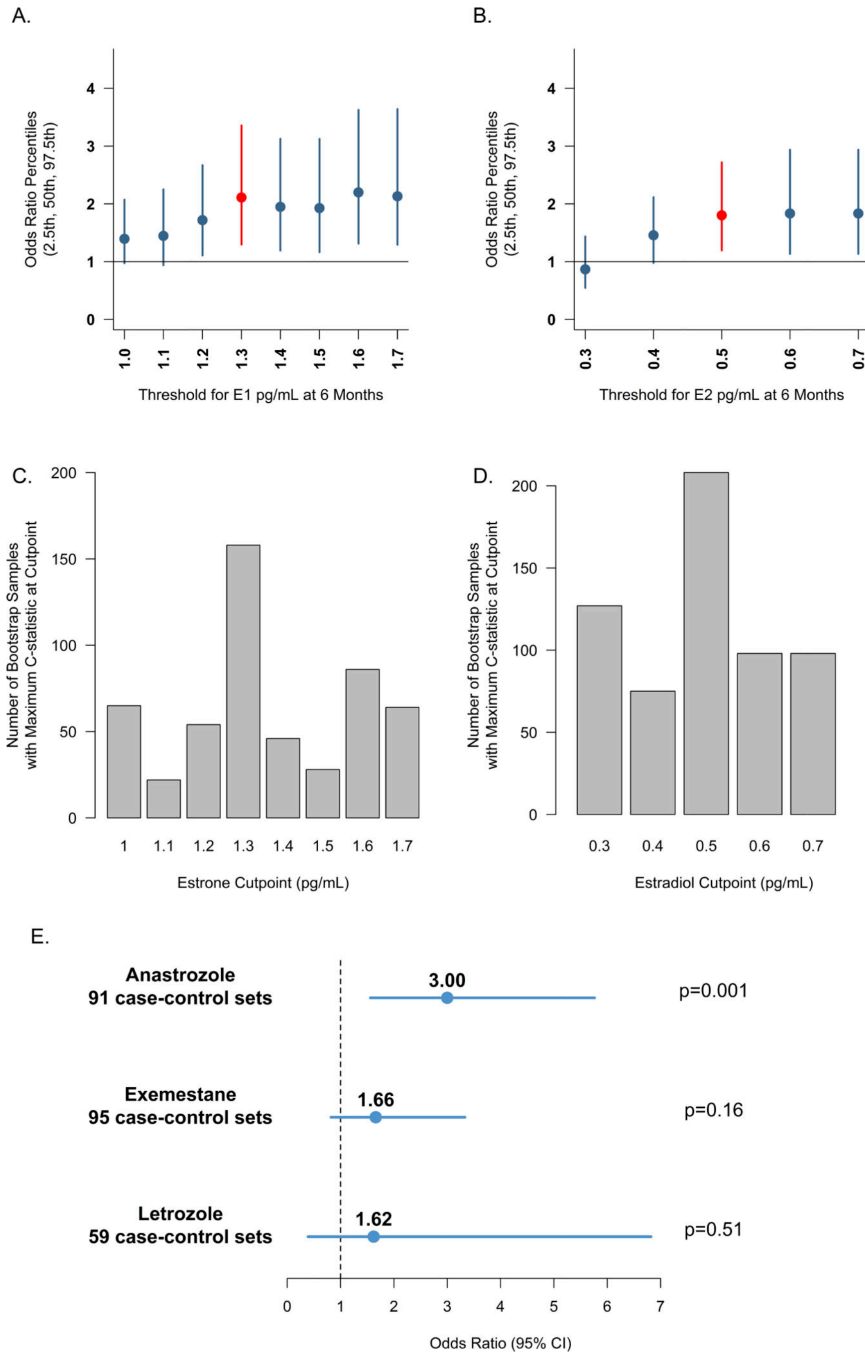

Figure 1.

Matched odds ratio percentiles derived from 500 bootstrap samples for the outcome of early breast cancer event according to thresholds for **A.** Estrone (E1) and **B.** Estradiol (E2) after six months of aromatase inhibitor therapy, analyzing anastrozole, exemestane, and letrozole together. **C**. Estrone (E1) and **D**. Estradiol: Barplots of bootstrap resampling results showing the number of samples (out of 500 bootstrap samples) for which each cutpoint had the maximum C-statistic discriminating between cases with early breast cancer event versus controls. Sum is greater than 500 due to ties. **E.** Summary of matched odds ratios for an

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early breast cancer event for anastrozole, exemestane, and letrozole analyzed separately, comparing patients with estrone (1.3 pg/mL) and estradiol (0.5 pg/mL) after 6 months of AI therapy to patients with estrone and/or estradiol below these thresholds.

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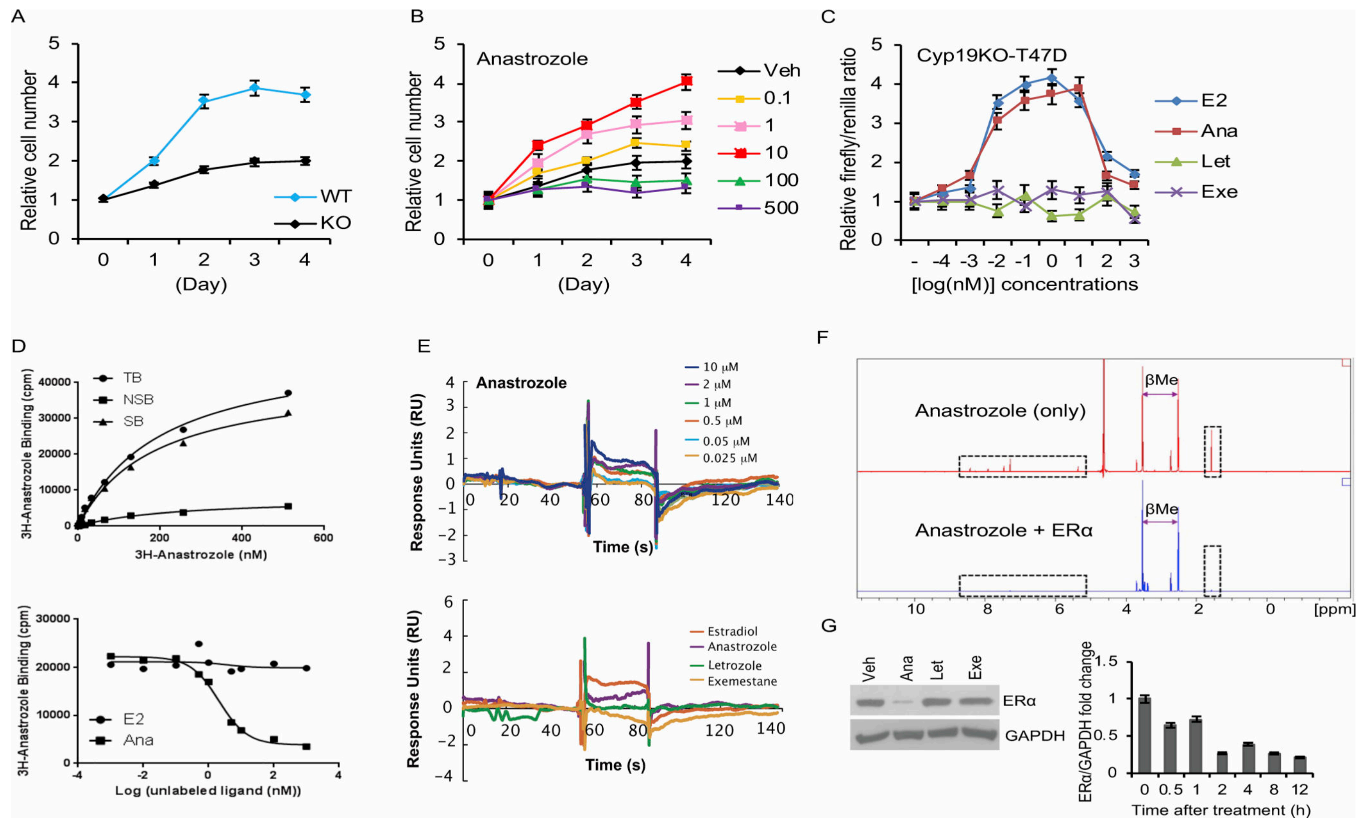

Figure 2.

Anastrozole binding to ERα. **A.** Cell growth of *CYP19A1* CRISPR knockout (KO) T47D and wild type cells. **B.** Cell growth of *CYP19A1* CRISPR KO T47D cells in the presence of anastrozole (0.1, 1, 10, 100, and 500 nM). **C.** Estrogen response element-dependent luciferase assay in *CYP19A1* CRISPR KO T47D cells treated with indicated concentrations of E2, anastrozole (Ana), letrozole (Let) or exemestane (Exe). **D.** Radioligand binding assays. Upper panel: [^{3}H]-anastrozole binding with ERα protein. TB: total binding, NSB: non-specific binding, SB: specific binding. Lower panel: [^{3}H]-anastrozole binding to ERα is competed off with increasing concentrations of cold anastrozole, but not with E2. **E**. Upper panel: ERα binding assays of anastrozole at different concentrations as indicated using a surface plasmon resonance biosensor. Lower panel: Comparisons among all four compounds at 2 μM. **F.** Anastrozole binding of ERα detected by nuclear magnetic resonance (NMR) spectroscopy. One-dimensional proton Carr-Purcel-Meiboom-Gill NMR spectroscopy of 120 μM anastrozole in the absence (top, red) and presence (bottom, blue) of 3 μM ERα protein. Dashed boxes showed decreased NMR signals. Spectra were normalized to each other using β-mercaptoethanol (βMe) signal shown in purple. **G.** Anastrozole and ERα degradation. Left panel: ERα protein levels in *CYP19A1* CRISPR KO T47D cells treated with 100 nM of each AI. Right panel: Western b quantification analysis show a time dependent decrease of ERα in *CYP19A1* CRISPR KO T47D cells treated with anastrozole.

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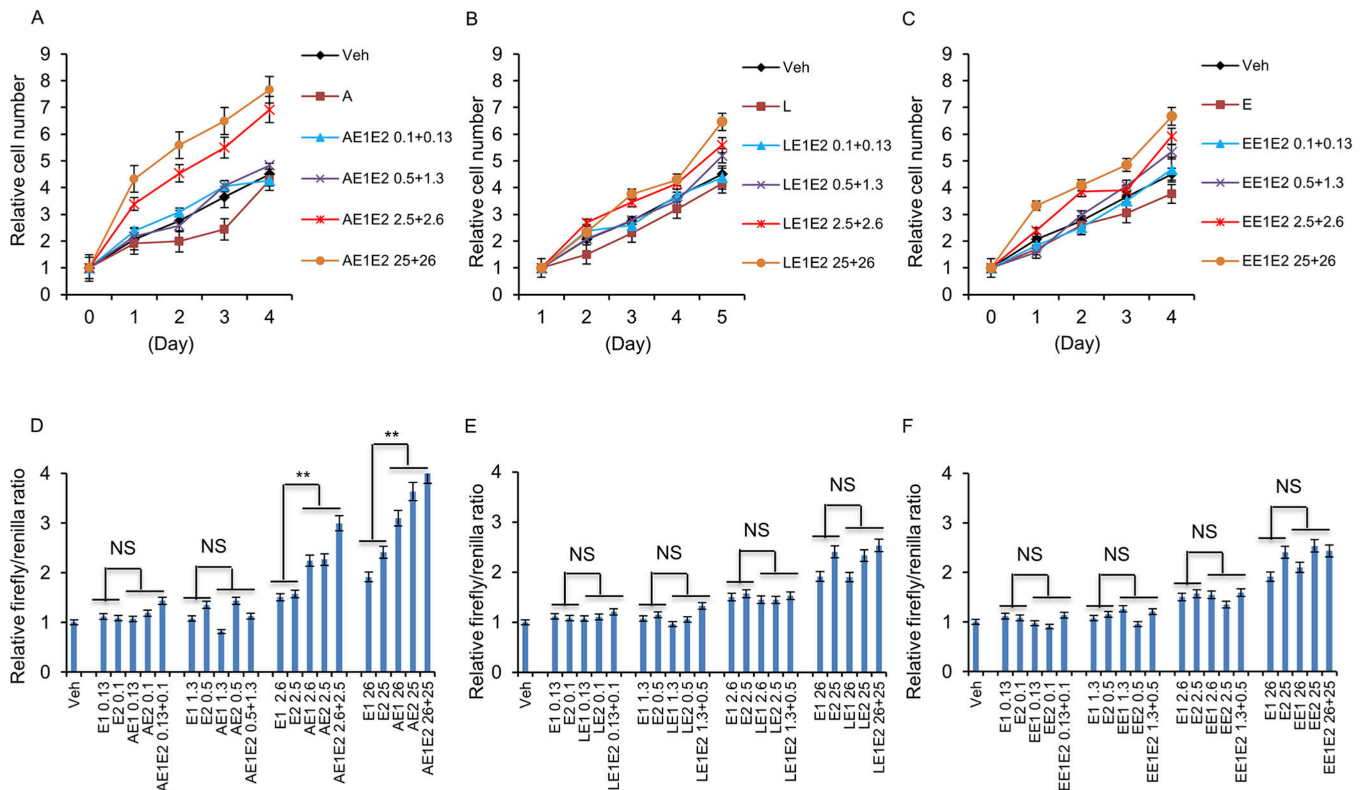

Figure 3.

Anastrozole potentiates estrogen effects when estrogen levels are above the thresholds. **A.** Cell growth of *CYP19A1* KO T47D cells in the presence of anastrozole (A) (27 ng/ml) plus indicated concentrations of estrone (E1) (0.13, 1.3, 2.6, and 26 pg/mL) and estradiol (E2) (0.1, 0.5, 2.5, and 25 pg/mL). **B.** Cell growth of *CYP19A1* CRISPR KO T47D cells in the presence of letrozole (L) (215 ng/mL) plus estrone (E1) and estradiol (E2). **C.** Cell proliferation of *CYP19A1* CRISPR KO T47D cells in the presence of exemestane (E) (10 ng/mL) plus estrone (E1) and estradiol (E2). **D-F.** Estrogen response element-dependent luciferase assay in *CYP19A1* CRISPR KO T47D cells treated with indicated concentrations of E1 (0.13, 1.3, 2.6, and 26 pg/mL), E2 (0.1, 0.5, 2.5, and 25 pg/mL), or in combination with anastrozole (A) (27 ng/mL), letrozole (L) (215 ng/mL), and exemestane (E) (10 ng/mL). Error bars represent the SEM of three independent experiments. **p<0.01.

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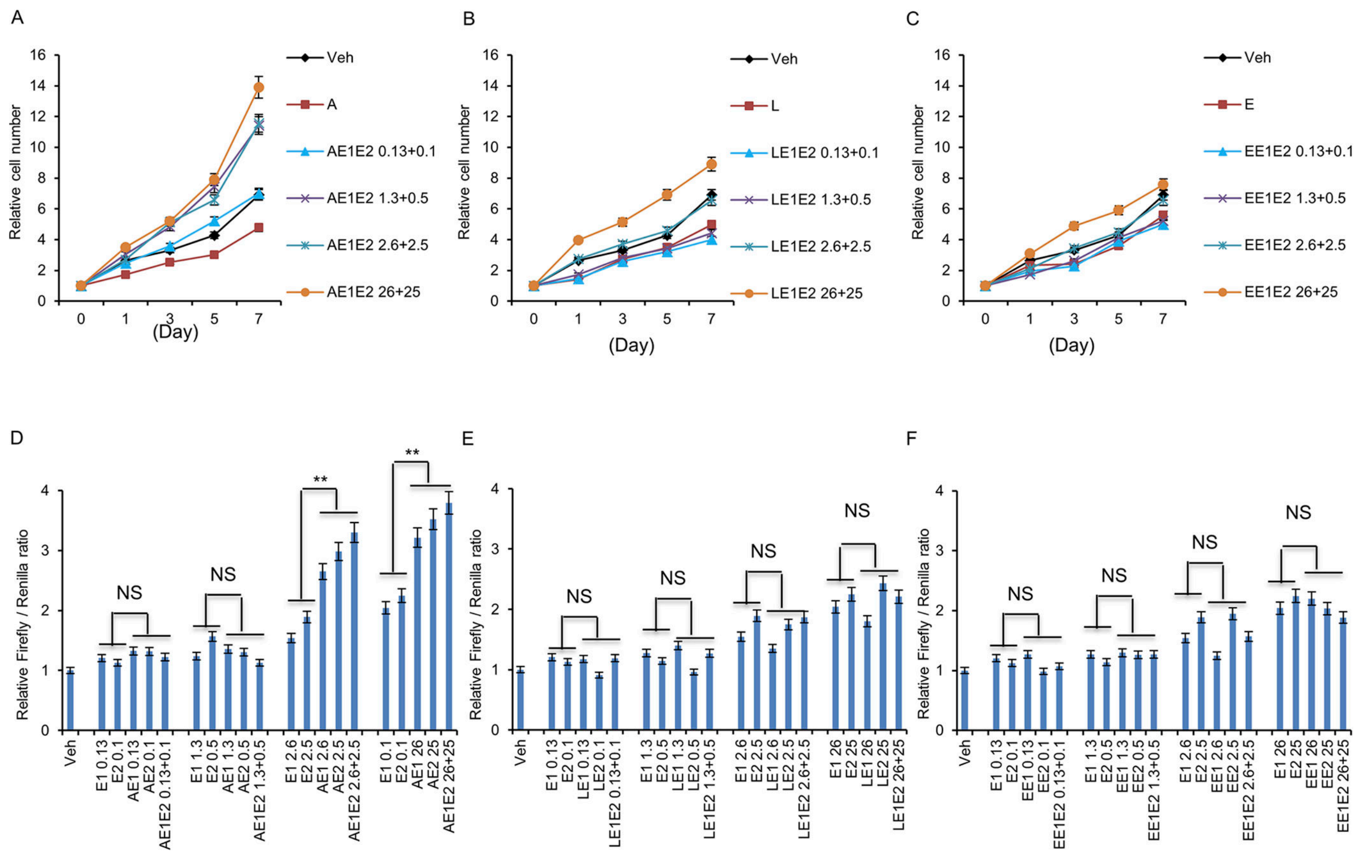

Figure 4.

Anastrozole potentiates estrogen effects when estrogen levels are above the thresholds. **A.** Cell proliferation of MCF7 cells in the presence of anastrozole (A) (27 ng/mL) plus indicated concentrations of estrone (E1) (0.13, 1.3, 2.6, and 26 pg/mL) and estradiol (E2) (0.1, 0.5, 2.5, and 25 pg/mL). **B.** Proliferation of MCF7 cells in the presence of letrozole (L) (215 ng/mL) plus indicated concentrations of estrone (E1) and estradiol (E2). **C.** Cell proliferation of MCF7 cells in the presence of exemestane (E) (10 ng/mL) plus E1 and E2. **D-F.** Estrogen response element dependent luciferase assay in MCF7 cells treated with the indicated concentrations of E1 (0.13, 1.3, 2.6, and 26 pg/mL), E2 (0.1, 0.5, 2.5, and 25 pg/mL) alone or in combination with anastrozole (A) (27 ng/mL), letrozole (L) (215 ng/mL), and exemestane (E) (10 ng/mL). Error bars represent the SEM of three independent experiments. **$p<0.01$.

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Table 1.

Characteristics by case-control status of patients included from the MA.27 and PreFace clinical trials.

	MA.27 cohort		PreFace cohort	
	Cases (n=186)	**Controls (n=327)**	**Cases (n=61)**	**Controls (n (n =171)**
Drug				
Anastrozole	91 (48.9%)	162 (49.5%)	0	0
Exemestane	95 (51.1%)	165 (50.5%)	0	0
Letrozole	0	0	61 (100%)	171 (100%)
Age, Median	63.8	64.0	61.5	62
Range	47.3–84.9	45.9–84.4	48.0–81.0	50.0–82.0
Race (self-reported)				
White	175 (94.1%)	309 (94.5%)	60 (98.4%)	171 (100%)
Black	7 (3.8%)	12 (3.7%)	0	0
Asian	1 (0.5%)	5 (1.5%)	0	0
Native American	0	1 (0.3%)	0	0
Hawaiian or Pacific Islander	1 (0.5%)	0	0	0
Not provided	2 (1.1%)	0	1 (1.6%)	0
Body Mass Index, Median	29.1	29.0	26.5	26.1
Range	17.5–56.8	18.3–55.4	17.6–45.2	17.6–47.3
< 25	45 (24.2%)	74 (22.6%)	26 (42.6%)	74 (43.3%)
25.0–34.99	113 (60.8%)	204 (62.4%)	30 (49.2%)	86 (50.3%)
35	28 (15.1%)	48 (15.0%)	5 (10.3%)	11 (6.4%)
Pathologic Stage				
I	51 (27.4%)	90 (27.5%)	21 (34.4%)	61 (35.7%)
II	85 (45.7%)	153 (46.8%)	27 (44.3%)	76 (44.4%)
III	50 (26.9%)	84 (25.7%)	13 (21.3%)	34 (19.9%)
Node positive				
No	83 (44.6%)	142 (43.4%)	33 (54.1%)	95 (55.6%)
Yes	103 (55.4%)	185 (56.6%)	28 (45.9%)	76 (44.4%)
ER/PR status				
ER+/PR+	136 (73.1%)	259 (79.2%)	55 (90.2%)	136 (79.5%)
ER+/PR−	44 (23.7%)	58 (17.7%)	5 (8.2%)	34 (19.9%)
ER+/PR unknown	4 (2.2%)	9 (2.8%)	0	0
ER−/PR+	2 (1.1%)	1 (0.3%)	1 (1.6%)	0
“Hormone receptor positive”	0	0	0	1 (0.6%)
Prior Adjuvant Chemotherapy	105 (56.5%)	185 (56.6%)	31 (50.8%)	87 (50.9%)
Events				
Local regional only	33 (17.7%)	0	5 (8.2%)	0

	MA.27 cohort		PreFace cohort	
	Cases (n=186)	Controls (n=327)	Cases (n=61)	Controls (n (n =171)
Contralateral only	29 (15.6%)	0	14 (23.0%)	0
Distant only	108 (58.1%)	0	36 (59.0%)	0
Distant & local regional	13 (7.0%)	0	4 (6.6%)	0
Distant, local regional & contralateral	1 (0.5%)	0	0	0
Distant & contralateral	2 (1.1%)	0	1 (1.6%)	0
Site not reported	0	0	1 (1.6%)	0

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Table 2.

Estrone, estradiol, and aromatase inhibitor concentrations (cases and controls combined) in MA.27 and PreFace sets.

	Anastrozole	Exemestane	Letrozole
Estrone (pg/ml)			
Pre-treatment	n=237	n=242	n=232
Median	28.0	26.0	28.0
Inter-quartile range	20.0, 38.0	20.0, 41.0	19.0, 39.5
Range	4.3–171.0	0.5*–123.0	0.5*–154.0
Below LLQ	0 (0%)	3 (1.2%)	11 (4.7%)
Six-month	n=252	n= 259	N = 227
Median	1.1	0.5*	0.5*
Inter-quartile range	0.5*, 1.6	0.5*, 1.3	0.5*, 0.5*
Range	0.5*–83.0	0.5*–57.0	0.5*–12.0
Below LLQ	104 (41.3%)	165 (63.7%)	180 (79.3%)
Percent change, n (%)			
90% Decrease	216 (91.1%)	214 (88.4%)	205 (90.3%)
0–89% Decrease	17 (7.2%)	27 (11.2%)	19 (8.4%)
Increase	4 (1.7%)	1 (0.4%)	3 (1.3%)
Missing**	16	18	5
Estradiol (pg/ml)			
Pre-treatment	n = 238	n = 243	n = 232
Median	4.8	4.6	4.8
Inter-quartile range	3.0, 7.3	3.1, 7.3	2.8, 7.8
Range	0.9*–104.0	0.15*–52.0	0.15*–44.0
Below LLQ	0	1 (0.4%)	9 (3.9%)
Six-month	n=253	n=260	n=230
Median	0.4	0.3	0.3
Inter-quartile range	0.3, 0.7	0.15*, 0.40	0.15*–0.40
Range	0.15*–49.0	0.15*–27.0	0.15*–10.0
Below LLQ	30 (11.9%)	92 (35.4%)	112 (48.7%)
Percent change, n (%)			
90% Decrease	139 (58.4%)	170 (70.0%)	167 (72.6%)
0–89% Decrease	94 (39.5%)	68 (28.0%)	51 (22.2%)
Increase	5 (2.1%)	5 (2.1%)	12 (5.2%)
Missing**	15	17	2
Aromatase inhibitor (ng/ml)	n=253	n=260	n=232
Median	27.2	2.1	214.9

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	Anastrozole	Exemestane	Letrozole
Inter-quartile range	20.2, 34.3	1.0, 4.5	169.1, 290.8
Range	0.03–82.5	0.07–38.0	2.4–559.6

LLQ: lower limit of quantitation.

*Represents values below the LLQ (1.0 pg/mL for E1 and 0.3 pg/mL for E2), which were analyzed using ½ the LLQ.

**Missing data in percent change measure is due to unavailable or unquantifiable values in the baseline or six month samples.