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Steroid Ligands, the Forgotten Triggers of Nuclear Receptor Action; Implications for Acquired Resistance to Endocrine Therapy

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ABSTRACT

Purpose: There is strong epidemiologic evidence indicating that estrogens may not be the sole steroid drivers of breast cancer. We hypothesize that abundant adrenal androgenic steroid precursors, acting via the androgen receptor (AR), promote an endocrine-resistant breast cancer phenotype.

Experimental Design: AR was evaluated in a primary breast cancer tissue microarray ($n = 844$). Androstenedione (4AD) levels were evaluated in serum samples ($n = 42$) from hormone receptor–positive, postmenopausal breast cancer. Levels of androgens, progesterone, and estradiol were quantified using LC/MS-MS in serum from age- and grade-matched recurrent and nonrecurrent patients ($n = 6$) before and after aromatase inhibitor (AI) therapy (>12 months). AR and estrogen receptor (ER) signaling pathway activities were analyzed in two independent AI-treated cohorts.

Results: AR protein expression was associated with favorable progression-free survival in the total population (Wilcoxon, $P < 0.001$). Pretherapy serum samples from breast cancer patients showed decreasing levels of 4AD with age only in the nonrecurrent group ($P < 0.05$). LC/MS-MS analysis of an AI-sensitive and AI-resistant cohort demonstrated the ability to detect altered levels of steroids in serum of patients before and after AI therapy. Transcriptional analysis showed an increased ratio of AR:ER signaling pathway activities in patients failing AI therapy (*t* test $P < 0.05$); furthermore, 4AD mediated gene changes associated with acquired AI resistance.

Conclusions: This study highlights the importance of examining the therapeutic consequences of the steroid microenvironment and demonstrable receptor activation using indicative gene expression signatures.

Introduction

Breast cancer subtyping is dominated by the steroid and growth factor receptor landscape with both estrogen receptor (ER) and progesterone receptor (PR) recognized as good prognostic markers (1). Expression of ER and/or PR protein supports the prescription of endocrine therapies, which selectively inhibit ER function or deplete estrogen synthesis. Currently, aromatase inhibitor (AI) medication is recommended as adjuvant first-line therapy for hormone receptor–positive, postmenopausal breast cancer (2).

Classic (genomic) nuclear receptor function arises from the binding of DNA and mediation of transcriptional programs, exemplified by the activation and selective recruitment of ER in response to binding by steroid ligands (3, 4). Often when considering the roles of sex steroids, we pigeon-hole them based on their masculinizing/feminizing effects. However, this understates the potency of these powerful, systemic signaling molecules that play important roles not only in reproduction and development but also circadian control, xenobiotic response, cancer, and basal and lipid metabolism (reviewed by ref. 5). In light of this, there is now renewed focus on alternate steroid facilitators of breast cancer progression (6, 7), nongenomic steroid action (8) and also members of the nuclear receptor subfamily 3C, which includes the androgen receptor (AR; refs. 9–11). In preclinical cell models, many studies have explored the differential impact of androgen agonists and antagonists often concluding that relative abundance of ER and AR is indicative of response. A number of insightful breast cancer studies have examined AR:ER protein ratio in clinical patient cohorts and concluded that a high level of AR:ER is indicative of poor response to traditional ER-targeting endocrine therapies (10, 12–14). AR agonists are often determined to antagonize pro-proliferative ER action in estrogen receptor positive (ER^+) androgen receptor positive (AR^+) tumors, whereas AR antagonists counteract the pseudo-ER role of AR in the triple-negative setting (15). This is reflective of the AR-targeting clinical trials scene in which agonists and antagonists all exhibit some degree of efficacy (reviewed in ref. 16). One confounding factor is the low threshold of AR positivity for inclusion in these trials (>1%); this will account for the majority of the patient cohort and is nondiscriminating. However, in all these studies, one vital element that is often overlooked is the influence of circulating steroids.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

It is now appreciated that changes in tumor gene and protein expression are influenced by age-dependent host endocrine factors as well as therapy-induced alterations in the steroid hormone milieu. By merging tumor androgen receptor (AR) protein expression and circulating steroid levels, we profiled patients with acquired resistance to aromatase inhibitor (AI) therapy. AR protein expression alone significantly associates with favorable progression-free survival in the total population ($n = 844$). However, steroid analysis of estrogen receptor (ER)-positive AI-sensitive and AI-resistant patient cohorts showed an ability to detect dynamic changes in circulating steroid levels in patients on AI treatment. Furthermore, acquired AI resistance associated with an increased ratio of AR:ER signaling pathway activities and androstenedione-associated gene changes. This study highlights the importance of examining the therapeutic consequences of the steroid microenvironment and demonstrable receptor activation using indicative gene expression signatures.

The concept of steroids as mediators of breast cancer development has been around for well over 100 years (17). Using modern techniques and newly gained proficiencies in steroid detection, this study takes a biochemical approach whereby steroid profiling in conjunction with functional signal transduction pathway analysis was carried out on breast cancer patient samples (18–20). This provides superior profiling compared with receptor protein expression alone. With chemical pathology moving away from radioimmunoassay toward more quantitative mass spectrometry approaches for steroid analysis, this timely research highlights the therapeutic potential of steroid profiling in postmenopausal breast cancers. Importantly, this study is the first to show dynamic response of circulating steroids to AI therapy and androgen-mediated gene changes associate with therapeutic response.

Materials and Methods

Patient samples—blood serum

Preoperative blood serum was collected from postmenopausal patients undergoing surgery for the resection of a clinically diagnosed primary breast tumor ($n = 42$). All patients were treated with adjuvant endocrine therapy. Blood samples were collected from each patient one day before surgical tumor removal. Preoperative blood serum was collected from hormone receptor–positive (ER, PR, AR), age-matched patients undergoing surgery for the resection of a clinically diagnosed primary breast tumor ($n = 6$). Subsequent follow-up serum samples were collected at scheduled intervals from these patients, all of whom went on to receive adjuvant AI therapy. Serum samples from the postoperative period were collected after a minimum of 12 months AI treatment. Clinical pathology data including ER and PR status, HER2 status, tumor grade, nodal status, and endocrine therapy were collated. The median follow-up period for the cohort was 52 months (minimum: 12 months, maximum: 96 months). See Supplementary Materials and Methods for detail on serum processing and storage.

Tissue microarray AR IHC

Mouse anti-human monoclonal AR primary antibody (AR-318-L-CE, Leica Biosystems) was used to detect AR in a previously constructed tissue microarray (TMA) of primary breast carcinomas (21). TMAs were scanned at 40× using a high-resolution digital scanner (Philips Digital Solutions) and the whole slide images were viewed remotely using QuPath software. Each primary tumor was represented by three individual tissue core specimens to ensure AR expression was reported as accurately as possible. Where discrepancies in calling occurred between cores the highest score was reported. Two individual, blinded scorers recorded data on cores and 30% of these were then validated by a third independent scorer. A histopathology (H-score) was used to evaluate expression levels of AR protein which comprises both percentage tissue expression and the intensity of the AR IHC stain. The scores were dichotomized based on a 75% cutoff (H-score %> 300) as recommended as a robust threshold in a recent publication by Ricciardelli and colleagues (22); this resulted in an AR-high (50% population) and AR-low (50% population) grouping.

Serum steroid analysis—androstenedione ELISA

A total of 25 μL of standards, controls, or serum samples were added to each well of the androstenedione (4AD) ELISA (Abcam, ab108672) microplate and protocol was carried out as per manufacturer's instructions.

Mass spectrometry

Steroids [4AD, 11-keto-testosterone (11KT), 11β-hydroxyandrostenedione (11OHA4), testosterone (T), 17-OH-progesterone (17OHP), and dehydroepiandrosterone sulphate (DHEAS)] were quantified in serum samples from patients with breast cancer ($n = 6$) using previously described LC/MS-MS methods (23, 24). The same samples were analyzed for 17β-estradiol (E_2) using LC/MS-MS, the details of the method and its performance characteristics are provided in Supplementary Data (Supplementary Materials and Methods).

Transcriptional analysis

OncoSignal (www.philips.com/oncosignal) assesses the activity of cell signal transduction pathways within tumors. In brief, for each signal transduction pathway (ER, AR) target genes have been identified. mRNA expression levels of these genes are translated into quantitative pathway activity scores (0–100) using a Bayesian network computational model (25). Formalin-fixed paraffin-embedded (FFPE) sections of postmenopausal ER/PR/AR-positive tumors from patients who went on to receive AI therapy were included in this study. Tumor sections were stained with hematoxylin and eosin and tumor specimens with epithelial content >70% were processed. Philips OncoSignal processed four 10-μm-thick FFPE sections of primary resected breast cancer for RNA extraction, and pathway activity analysis. AR, ER, and PI3K pathway activity was then reported as a numerical value between 0 and 100 with 95% confidence interval (CI).

Genomic AR (26, 27) and ER signaling markers were also assessed in an independent postmenopausal breast cancer cohort. Gene expression data from sequential samples treated with estrogen deprivation therapy (GSE111563, GSE59515, GSE55374, and GSE20181) were used in the analysis. (28, 29). Gene expression was given relative to pretreatment. Previously published 4AD-driven gene expression was profiled in dormant and acquired resistant patients using these datasets (7).

Statistical analysis

Detailed clinical information was available for the primary breast cancer tumor samples run on the TMA ($n = 844$). A t test was used to compare the ages and tumor sizes of patients between the AR-high and AR-low groups (after testing for normality with the Shapiro–Wilks test). A Fisher exact test was used to compare all the other clinical parameters examined for differences between the AR-high and

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AR-low groups. For survival analysis, the data were dichotomized on the basis of AR high and AR low. Univariate Kaplan–Meier analysis was performed using both overall survival (OS) and progression-free survival (PFS) as the survival endpoints. Multivariate survival analysis was performed using Cox Regression analysis with ER, PR, tumor grade (< grade 3 vs. grade 3), lymph node status, tumor size (< 25 mm vs. ≥ 25 mm), and patient age (< 52 years old vs. ≥ 52 years old) as covariates. Spearman rho was used to assess the relationship between 4AD and age in the age- and grade-matched subset of samples (these were not normally distributed). Linear regression was also used to assess the relationship between 4AD and age (in this case, the data were log transformed to meet the assumptions of a linear model). Where appropriate, P values were adjusted using the Benjamini–Hochberg method. For transcriptional analysis, gene expression was given relative to pretreatment. Statistical comparisons were performed using two-tailed unpaired Student t test and ANOVA followed by Tukey multiple comparison, $P < 0.05$ was considered as significant. All statistical analysis was performed in the R environment (https://www.r-project.org/) and Bioconductor.

Ethics and consent

Written and informed consent was acquired prior to collection of patient tumor tissue under The Royal College of Surgeons Institutional Review Board–approved protocol (#13/09:CTI 09/07). All Czech patients were informed about the study and those who agreed and signed an informed consent participated in the study. Study protocol was approved by the Ethical Commission of the National Institute of Public Health in Prague (approval nos. 6715-3, 9799-4, and 15-25618A). Studies were conducted in accordance with the Declaration of Helsinki.

Results

AR protein expression and patient survival in a TMA of primary breast cancer

To evaluate whether the bioavailability of androgens in the AI-resistant setting impacts the role of AR, we first stained a cohort of primary breast tumors for AR protein expression. The TMA cohort had data on a range of clinical parameters including ER, PR, HER2 status, tumor grade, nodal status, age (<52>) and whether the patients received tamoxifen or AI therapy (**Table 1**). A H-score threshold of 75% staining intensity (H-score 300) was applied to dichotomize the cohort into AR low (<300) and AR high (=> 300; **Fig. 1A**). AR IHC was graded using a H-score ranging from 0 to 400 with representative images highlighting the predominantly nuclear localization including its expression in normal ductal structures [**Fig. 1B (i–vi)**]. Survival analysis indicated that high levels of AR (H-score 300) confers a PFS benefit in the overall population ($P < 0.001$) [**Fig. 1C (ii)**], however, when patients are stratified into those that have been treated with endocrine therapy this protective effect is no longer apparent [**Table 2**; Supplementary Fig. S1 (i–vi)]. Univariate analysis of high AR expression in the total population ($n = 654$) showed significant association with improved PFS (HR: 0.59; 95% CI: 0.39–0.88; **, $P < 0.01$). Of note, patients treated with AI ($n = 205$) exhibited an increased risk (HR = 1.6); however, this was not significant. A similar trend was also observed for OS with AI monotherapy resulting in HR = 1.2 in contrast to the lower HR of either tamoxifen alone or as combination therapy with AI (HR = 0.69 and HR = 0.4, respectively; **Table 2**). When we focus on the impact of high AR across ER^+, $HER2^+$, and triple-negative type cancers, we find AR to associate with improved PFS in ER^+ cancer (**, $P = 0.002$; HR: 0.48; Supplementary Table S1). Conversely, high AR associates with poor outcome in triple-negative breast cancers (*, $P = 0.013$; HR: 3.27) [**Fig. 1C (vi)**], in addition, no significant impact on the $HER2^+$ population was noted (Supplementary Table S1). Multivariate analysis showed that only an ER^+ status provided significant risk reduction in OS (HR: 0.48; 95% CI:0.26–0.88; *, $P = 0.02$) with high tumor grade, positive nodal status, and postmenopausal age associating with increased risk. With regard to PFS, only positive nodal status was found to significantly associate with increased risk of recurrence (HR: 2.14; 95% CI: 1.34–3.43; **, $P = 0.002$; Supplementary Table S2).

Table 1. Clinical and pathologic parameters and their association were evaluated by Student t test (age, tumor size) and by Fisher exact test [node, grade, ER^+, PR^+, lymphovascular invasion (LVI), tamoxifen and AI therapy].

Clinical parameters	Total n	AR Low (Q1–Q3) %	AR High (Q4) %	P_{adj}
Age ≤ 52	317	54	46	**$<2 \times 10^{-16}$**
>52	366	47	53	**$<2 \times 10^{-16}$**
Tumor size ≤ 2.5 cm	546	50	50	0.08
>2.5 cm	113	51	49	0.08
Tumor grade < 3	441	40	60	—
= 3	217	71	29	**4×10^{-13}**
Node +VE	372	51	49	—
−VE	276	49	51	0.75
Estrogen receptor +VE	540	42	58	—
−VE	125	84	16	**$<2 \times 10^{-16}$**
Progesterone receptor +VE	414	38	62	—
−VE	252	70	30	**9.06×10^{-15}**
HER2 +VE	101	66	34	—
−VE	563	47	53	[a]**0.001**
LVI +VE	314	52	48	—
−VE	348	48	52	0.34
Tamoxifen +VE	273	45	54	—
−VE	230	43	57	0.79
Aromatase inhibitor +VE	271	41	59	—
−VE	229	48	52	0.14

Note: Values in bold are statistically significant.
[a]Denotes inverse association.

Assessment of sex steroids in patients with postmenopausal breast cancer and in a subset of AI-sensitive and AI-resistant blood serum samples

Many epidemiologic studies have reported association between sex steroid levels and increased risk of breast cancer. That said, there are also many inconsistencies reported between levels and type of steroids present, and associated risk (30). We have previously shown that elevated levels of 4AD-driven transcriptional changes associated with poor response to endocrine therapy (7). A cohort of breast cancer serum samples from patients (recurrent and nonrecurrent to endocrine therapy, $n = 42$) were evaluated for 4AD using an ELISA. The 4AD values for eight recurrent and one nonrecurrent sample lay outside the reference range for 4AD in postmenopausal women (ref. 31; Supplementary Fig. S2A). When these serum levels were plotted by age at primary diagnosis and grouped by decade, there was a broader spread in the levels detected within the endocrine recurrent cohort (**Fig. 2A**). A proportion of these samples were age and grade matched to adjust for the greater number of recurrent specimens. When these data were plotted versus increasing age, it was noted that there was a strong inverse

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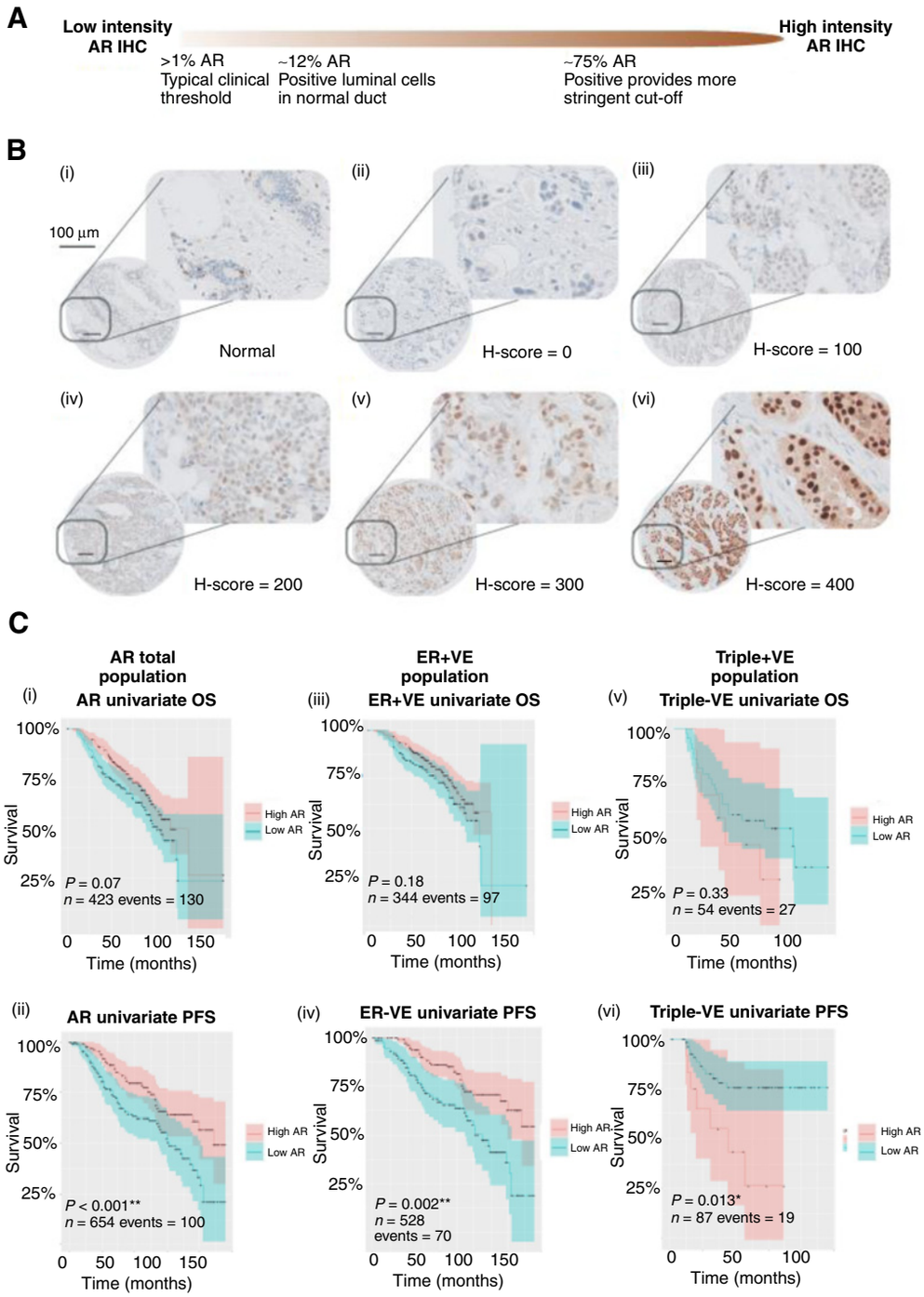

Figure 1.

Survival analysis of a TMA of primary breast cancer stained immunohistochemically for AR ($n = 844$). **A,** Graphical representation of common cut-off points in AR IHC studies with the bulk of clinical data applying an AR protein expression >1% to denote a positive stain. Normal breast ducts are reported to express approximately 12%. A more stringent cut-off point of 75% has been reported, and this was applied to the current study. **B,** AR protein expression was recorded as H-score (0–400) with a cut-off point of H-score = 300. Representative TMA cores of AR staining in normal breast tissue are depicted (i), alongside images of tumor staining of AR ranging in intensity from 0 to 400 (ii–vi). **C,** (i), Kaplan–Meier of OS univariate analysis of AR in the total population, log rank $P = 0.07$. (ii), Kaplan–Meier of PFS of the same population, log rank $P = 0.001$. (iii), Kaplan–Meier of OS univariate analysis of AR in the ER population, log rank $P = 0.18$. (iv), Kaplan–Meier of PFS of the same population, log rank $P = 0.002$. (v), Kaplan–Meier of OS univariate analysis of AR in the triple negative population, log rank $P = 0.33$. (vi), Kaplan–Meier of PFS of the same population, log rank $P = 0.013$.

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Table 2. Univariate analysis of AR high (H-score ≥75%) showing HRs in the total population, tamoxifen alone, AI alone, or combination tamoxifen and AI-treated patients.

Univariate: Androgen Receptor HIGH			
Clinical parameter	**HR**	**95% CI**	***P***
Progression-free survival $n = 654$	0.59	0.39–0.88	**0.001**
AI therapy $n = 205$	1.60	0.68–3.78	0.29
Tamoxifen therapy $n = 202$	1.08	0.36–3.29	0.9
AI and tamoxifen $n = 40$	1.36	0.48–3.88	0.56
Overall survival $n = 423$	0.72	0.51–1.02	0.07
AI therapy $n = 151$	1.21	0.69–2.12	0.51
Tamoxifen therapy $n = 117$	0.69	0.27–1.78	0.45
AI and tamoxifen $n = 40$	0.40	0.17–1.26	0.12

Note: Value in bold is statistically significant.

correlation (**Fig. 2B**; rho = −0.79, $P = 0.048$) with decreasing levels of 4AD with age in the nonrecurrent patients (**Fig. 2B**), this degree of correlation was not observed for the recurrent population (**Fig. 2B**; rho, −0.52, n.s.).

To provide greater clarity, we then looked at pre- and post-therapy serum steroid levels in a cohort of patients who were recurrent on AI treatment, and patients who were classified as nonrecurrent with a median follow-up time of 52 months. All recurrent and nonrecurrent patient primary tumors were matched by age (ages: 49–49, 66–68, 71–76), all were positive for AR, ER, and PR and were HER2 negative. The specific effect of AI therapy resistance on circulating steroids was investigated using a LC/MS-MS to assess a panel of steroids in sequential serum samples of these patients. To this end, the levels of E_2, T, 4AD, 11-kT, 11OH4A, and 17OHP were measured in an AI-treated cohort ($n = 6$) [**Fig. 3A**; Supplementary Fig. S2 (B (i–vii)); Supplementary Table S3]. Reflective of the postmenopausal patient population, estradiol was detected at low picomolar (<10 pmol/Lol) levels in the majority of patients. Of note, 2 of the 3 AI nonrecurrent patients had detectable levels of E_2 pretreatment, which was completely ablated posttreatment, demonstrating the efficacy of AI therapy in suppressing estradiol synthesis (Supplementary Table S3). Furthermore, 17OHP levels were unaltered pre- and post-AI therapy regardless of clinical response to the therapy (Supplementary Table S3). Conversely, androgenic steroids; DHEAS, 4AD, T, 11KT, and 11OHA4 levels trended toward higher levels pre- and post-AI therapy in the recurrent patients compared with those who were nonrecurrent (**Fig. 3A**; Supplementary Table S3).

Dynamic changes in circulating steroid levels are evident in patients on AI treatment

To determine whether it is possible to quantify alterations in steroid levels while patients are on treatment, we compared matched steroid concentrations pre- and post-AI therapy in recurrent and nonrecurrent patients [**Fig. 3B (i–vi)**]. While there was no significant difference in the mean levels of 4AD in the pretreatment samples [**Fig. 3B** (i)], assessment of 4AD levels focusing on post-AI treatment showed a trend toward increased 4AD in patients who recurred when compared with patients who responded to AI therapy [**Fig. 3B** (ii), *t* test $P = 0.018$, $P_{adj.} = 0.11$]. Two of 3 AI recurrent patients showed elevated levels of T compared with nonrecurrent patients pre-AI therapy [**Fig. 3B** (**iii**) *t* test $P = 0.32$, $P_{adj.} = 0.39$], and T levels do not appear to be influenced by AI treatment [**Fig. 3B** (**iv**) *t* test $P = 0.08$, $P_{adj.} = 0.25$]. 4AD can be converted to 11OHA4, which is unique to the adrenal glands. Therefore, studies suggest 11OHA4 can be used as an indicator of adrenal androgen secretion in women (23). There was no significant change in 11OHA4 in either the pre- or post-AI treatment groups but 2 of the 3 recurrent patients showed an approximately 2-fold increase in levels hinting at steroid production of adrenal origin [**Fig. 3B (v and vi)**] *t* test $P = 0.26$, $P_{adj.} = 0.39$ and *t* test $P = 0.21$, $P_{adj.} = 0.39$, respectively]. It was also noted that the overall androgen profile of patients with recurrent disease was much more disperse than those who responded to therapy [Supplementary Fig. S2B; recurrent (i–iii) and nonrecurrent (iv–vi)].

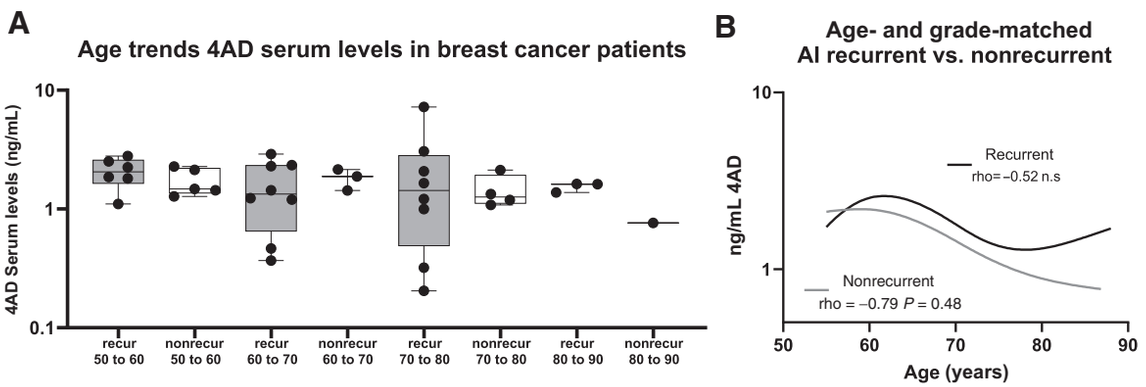

Figure 2.

Patients who have disease recurrence on endocrine therapy have a noisier serum androgen profile. **A,** Measured using ELISA serum 4AD levels of recurrent ($n = 25$) and nonrecurrent ($n = 13$) patients were plotted by age at primary diagnosis and grouped by decade. **B,** Log-transformed data of 4AD levels measured by ELISA of age- and grade-matched patients with recurrent and nonrecurrent breast cancer on endocrine therapy ($n = 17$). Nonrecurrent patient serum 4AD levels have a strong inverse association with increasing age (Spearman rho = −0.79, $P < 0.5$). Recurrent samples do not exhibit the same degree of correlation with age (Spearman rho = −0.5, n.s). Multivariate analysis interaction term $P = 0.09$.

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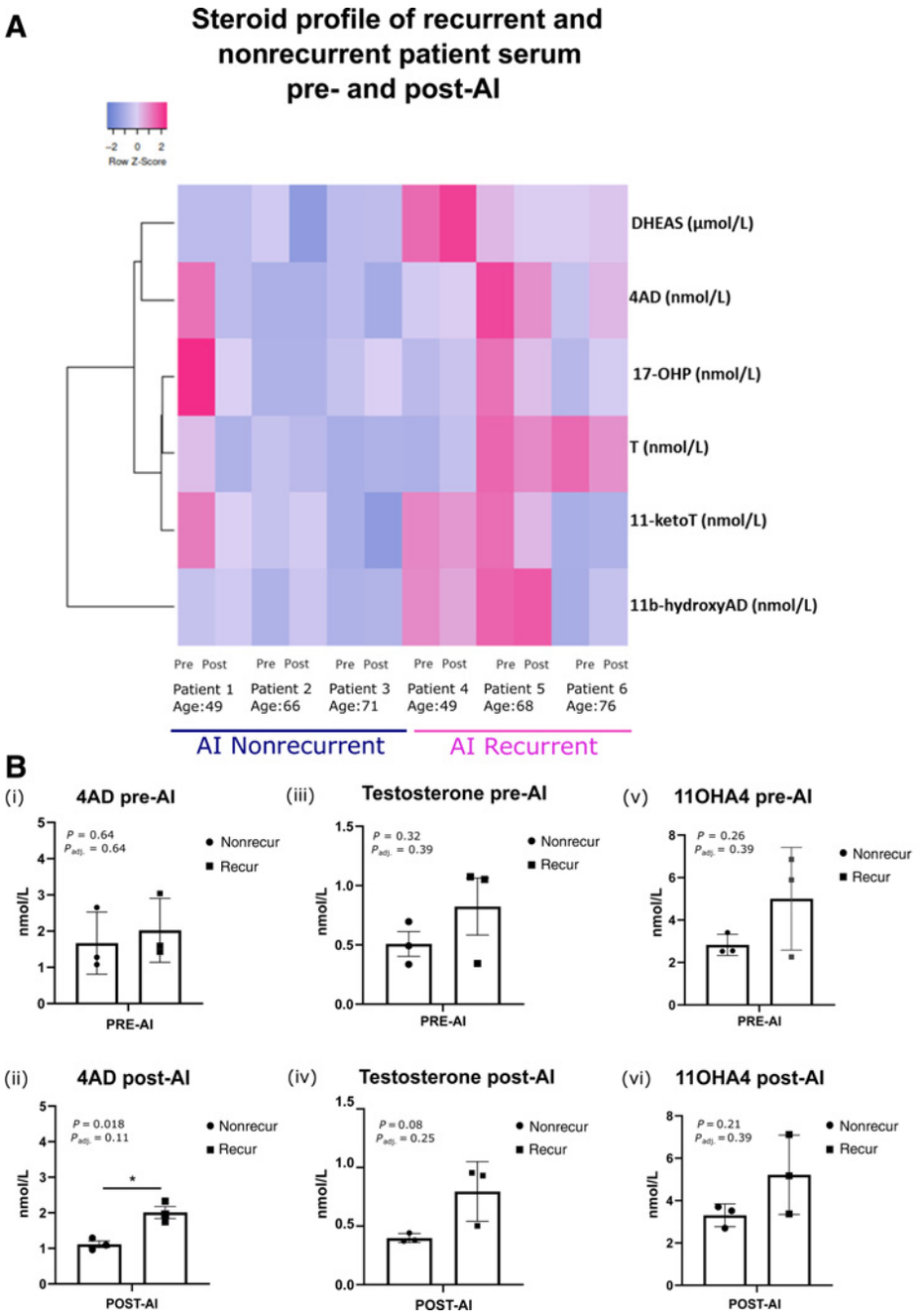

Figure 3.

Androstenedione is increased in recurrent breast cancer patient serum whilst on AI therapy. **A,** Heatmap (http://heatmapper.ca/expression/) with hierarchical clustering (Euclidian) representing pre- and post-AI patient serum levels (nonrecurrent: $n = 3$; recurrent $n = 3$) for the following steroids: DHEAS (μmol/L), 4AD (nmol/L), 11-KetoT (nmol/L), T (nmol/L), 11b-hydroxyAD (nmol/L), 17OHP (nmol/L; raw data: Supplementary Table S3). **B,** Changes in steroid levels before and after AI therapy comparing nonrecurrent and recurrent specimens. (i)—(ii), 4AD did not significantly alter pre- or post-AI among nonrecurrent patients (t test $P = 0.64$, $P_{adj.} = 0.64$), but there was an increase after AI in recurrent patients (t test $P = 0.018$, $P_{adj.} = 0.11$). (iii)—(iv), T did not significantly alter pre (t test $P = 0.32$, $P_{adj.} = 0.39$) or post (t test $P = 0.08$, $P_{adj.} = 0.25$) AI among recurrent and nonrecurrent patients. (v)—(vi), 11OHA4 in patients pre (t test $P = 0.28$, $P_{adj.} = 0.39$) or post (t test $P = 0.21$, $P_{adj.} = 0.39$) AI among recurrent and nonrecurrent patients.

Patients who have recurrence on AI therapy display higher AR-to-ER transcriptional activity

The detection of nuclear steroid receptor mRNA or protein does not always signify that they are transcriptionally active. To address the role of AR in AI-resistant patients more comprehensively, and in light of the elevated levels of 4AD-detected post-AI treatment, we carried out a signal transduction pathway activity analysis (OncoSignal) in collaboration with Philips Molecular Pathway Dx ($n = 3$ recurrent, $n = 3$ nonrecurrent, AI-treated patients). This technology quantitatively measured the expression and functionality of ER and AR in patient tissue. Age- and grade-matched breast cancer samples from AI-sensitive and recurrent patients were identified, the expression level of AR protein was evaluated by IHC and found to be readily detectable in all samples (Supplementary Fig. S3 for representative AR IHC staining). Results from our cohort of patients demonstrated active signaling in agreement with a luminal A subtype although, especially for the AR pathway activity, marked differences are observed between patients. A higher ratio of AR to ER pathway activation was detected in AI recurrent compared with nonrecurrent patients (t test, $P = 0.05$) [**Fig. 4A (i)**]. Pre-AI therapy serum samples from these patients were also analyzed for 4AD and while there was no significant difference in steroid between the recurrent and nonrecurrent cohort, there was a trend toward higher levels in the recurrent group [**Fig. 4A (ii)**]. To further validate these findings, we looked at AR and ER genomic

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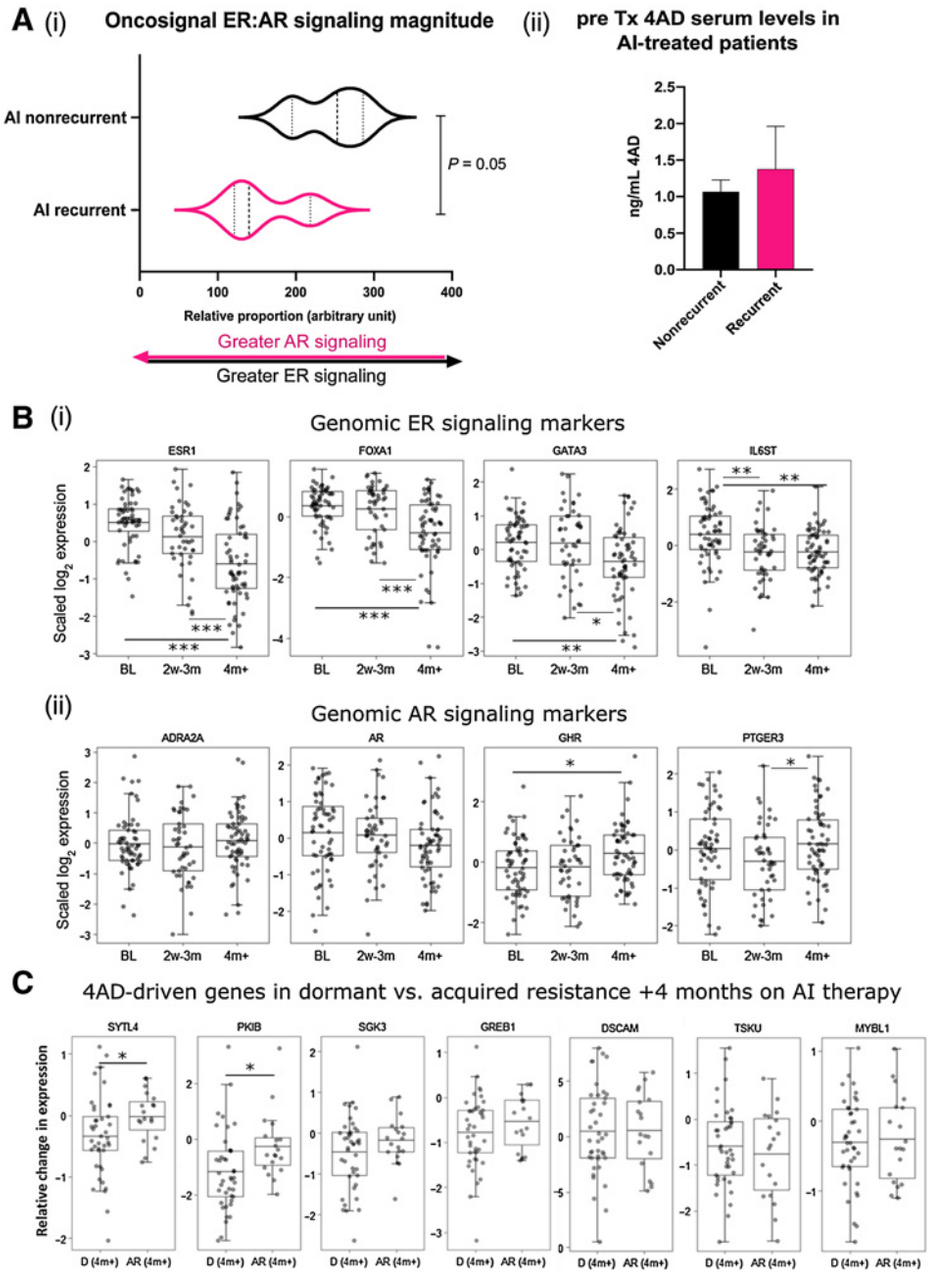

Figure 4.

Patients who have recurrence on AI therapy have greater AR versus ER pathway signaling in contrast to matched nonrecurrent patients. **A,** (i), Philips Oncosignal platform was used to assess functional activity of AR and ER signal transduction pathways. Ratios of gene pathway activation were evaluated in age- and stage-matched AI-responsive ($n = 3$) and AI-resistant breast cancers ($n = 3$). (ii), Matched 4AD serum levels from the patients were evaluated in pre-AI therapy serum samples via ELISA ($n = 6$). **B,** In an independent AI-treated clinical cohort, genomic ER (ESR1, FOXA1, IL6ST, GATA3) and AR signaling markers (AR, GHR, ADRA2A, PTGER3) were evaluated at timepoints—2 weeks, 3 months, 4 months ($n = 167$). **C,** 4AD-associated transcriptional alterations were evaluated in the AI-treated cohort separated into dormant and acquired resistance groupings ($n = 62$). *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$.

signaling markers in an independent previously published postmenopausal breast cancer cohort. Patients received AI therapy for a minimum of 4 months and changes in gene expression upon treatment in patient-matched on-treatments versus pretreatment was assessed by RNA analysis. In line with our previous findings, genomic ER signaling was observed to decrease over the duration of treatment; however, in contrast AR signaling was sustained (**Fig. 4B**). All ER signaling markers were significantly decreased from baseline to 4+months AI therapy (ESR1 $P < 0.0001$, FOXA1 $P < 0.0001$, GATA3 $P < 0.001$, IL6ST $P < 0.001$), whereas the breast cancer–specific AR signaling markers were increased or unchanged (GHR $P < 0.01$, PTGER3 $P < 0.01$, ADRA2A n.s, AR n.s). Using our previously published 4AD-regulated gene set (7), we further found that PKIB ($P < 0.01$) and SYTL4 ($P < 0.01$) were elevated in patients whose tumors developed acquired resistance compared with dormant tumors (**Fig. 4C**). Of note, four of the previously identified 4AD-driven genes PKIB, MYBL1, SYTL4, and DSCAM were only present +4 months post-AI therapy (Supplementary Fig. S4).

Discussion

Elevated androgens associate with both hormone receptor–positive and hormone receptor–negative breast cancers; highlighting the uncertainty around the pro-tumorigenic potential of steroids in the absence of their recognized receptors (32). Moreover, there is strong epidemiologic evidence inferring that estrogens may not be the only

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steroid drivers of breast cancer (33–35). We have previously shown that the androgenic steroid environment that results from AI therapy in breast cancers induces AR-mediated gene changes associated with poor response to conventional estrogen/ER-targeting therapies (7, 36). The data presented here highlight the importance of fully elucidating the role that circulating sex-steroid precursors and subsequent activation of the receptor is exerting on breast tumor survival in the setting of anti-estrogen therapy failure. While the concept of bioavailable androgens driving endocrine resistance is not new (32, 37, 38), this is the first study to show dynamic changes in steroid levels on treatment using sensitive mass spectrometry quantification of estrogen and androgens.

In this study, we have revisited AR IHC and breast cancer risk and applied a more stringent (IHC >75%) cut-off point (17). The uncertainty surrounding AR as a prognostic and/or predictive factor in the setting of endocrine-treated breast cancer was addressed by a recent analysis of the BIG 1-98 trial data (39). The authors determined that AR protein expression (IHC >1% scored positive) is not prognostic in the setting of hormone receptor–positive, postmenopausal breast cancer. They did, however, observe in patients assigned to letrozole monotherapy that AR expression associated nonsignificantly with poorer disease-free interval (HR = 1.52). This trend toward reduced disease-free interval in an AI-treated cohort is mirrored in our own study. Here we have shown elevated AR protein denotes a survival advantage that cannot be attributed to conventional subtyping (ER^+), nor is it associated with favorable response to endocrine therapy.

In our previous studies, we developed AI-resistant cell models cultured in 4AD to recapitulate the inhibition of estrogen synthesis (7, 36, 40). Transcriptional changes associated with 4AD exposure were also determined to strongly associate with poor response to AI therapy (7). While 4AD is not a potent androgen, it has been shown to be the most abundant intratumoural steroid in both breast cancer and castrate-resistant prostate cancer tissue (30, 41). To look at this clinically, we screened a cohort of ER^+, postmenopausal breast cancer sera. The data showed 4AD to be higher in the recurrent population with lower 4AD associated with increasing age only in the patients who responded to endocrine therapy. As ligand bioavailability may have major consequences for response to endocrine therapy, this prompted us to characterize the steroid landscape in a cohort of AI-responsive and AI-nonresponsive breast cancers. Evidence that combining steroid serum data to standard clinical pathology in breast cancer risk prediction has some precedence with a recent nested case–control study showing improved discrimination for estrogen receptor–positive disease (42). In our study, we found that we could detect changes in circulating androgens in patients whose breast cancers recurred while on AI therapy.

AR is reported to block the tumorigenic potential of ER, it therefore makes sense that ER^+/AR^+ tumors do better and this is widely reported in clinical meta-analysis, and which we too also show (39, 43). However, under AI treatment, the survival advantage is diminished, perhaps because AR is no longer acting in opposition to active ER signaling. To investigate whether this is the case, we evaluated AR and ER signaling pathways based on primary breast tumor gene expression analysis using Philips Oncosignal platform. While activity of signaling pathways for both steroid receptors were evident, this analysis showed a preponderance of AR signal transduction pathway activity over that of ER in patients who recurred on AI therapy. Further analysis of an independent AI-treated clinical cohort showed that while active ER gene markers diminished, breast cancer–specific AR signaling markers were increased or were static. Of note, 4AD-associated gene changes showed increased expression in acquired AI resistance versus dormant tumors, indicating that tumors with plentiful steroid precursors may adapt to long-term alteration of the steroid environment. These findings are in line with the recently published, phase II clinical trial, which showed that patients with high levels of AR mRNA and low levels ESR1 mRNA derived significant benefit from the combination of enzalutamide and exemestane (44). A possible explanation for this data is that diminished genomic activation of both ER and AR may be more permissive of non-canonical pathways. The reduced production of potent sex steroids, accompanied by a surfeit of weak precursor steroids in aging adults provides rationale for the benefit of therapeutic agents that drive genomic nuclear receptor activity (45, 46).

Despite breast cancer subtype classification being based on the fairly ubiquitous presence of hormone receptors (ER, PR); the presence of ligands are somewhat overlooked. The relevance of age-related endocrine changes in driving transcriptomic and proteomic alterations in breast cancer has recently garnered attention, particularly with regards the robustness of biomarker assessment platforms such as OncotypeDX (47, 48). This is a burgeoning area of research and it is clear that inclusion of the patient endocrine landscape may enhance robustness in the application of biomarker-based algorithms (47). In the past few years, there has been a shift toward LC/MS-MS detection of steroids with unparalleled specificity and sensitivity. This has revolutionized our capacity to quantify individual patient steroid levels and evaluate the relevance of these to patient response on endocrine therapy (30, 41). This study assessed systemic steroid levels, as although intratumoural steroid levels would be very informative the former is more clinically feasible. A limitation of this study is the small sample size; however, this was mitigated by age, steroid receptor, grade, and treatment matching of clinical samples. With precision oncology moving into mainstream clinical decision making, this study highlights the value of layering multiple components of the steroid tumor microenvironment. Steroid receptors, their ligands and signal transduction pathway activity profiling should all be factored in determining optimal treatments for hormone receptor–positive, postmenopausal breast cancer. Our study very importantly shows that it is possible to quantitatively detect circulating steroids in patient serum while on therapy.

Authors' Disclosures

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Authors' Contributions

R. Bleach: Data curation, validation, visualization, writing–original draft, writing–review and editing. **S.F. Madden:** Formal analysis, visualization, methodology, writing–review and editing. **J. Hawley:** Formal analysis, validation, methodology, writing–original draft, writing–review and editing. **S. Charmsaz:** Formal analysis, visualization, methodology, writing–review and editing. **C. Selli:** Data curation, formal analysis, validation, visualization, methodology, writing–review and editing. **K.M. Sheehan:** Formal analysis, supervision, visualization, methodology, writing–review and editing. **L.S. Young:** Resources, data curation, funding acquisition, writing–review and editing. **A.H. Sims:** Data curation, formal analysis, validation, visualization, methodology, writing–review and editing. **P. Souček:** Resources, data curation, methodology, writing–original draft. **A.D. Hill:** Resources, data curation, methodology, writing–review and editing. **M. McIlroy:** Conceptualization, formal analysis, supervision, funding acquisition, visualization, methodology, writing–original draft, Project administration, writing–review and editing.

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Steroid Ligands, the Forgotten Triggers of Nuclear Receptor Action; Implications for Acquired Resistance to Endocrine Therapy

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