

## Effect of Aromatase Inhibition on Functional Gene Modules in Estrogen Receptor–Positive Breast Cancer and Their Relationship with Antiproliferative Response

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### Abstract

**Purpose:** To investigate potential associations between gene modules representing key biologic processes and response to aromatase inhibitors (AI) in estrogen receptor–positive (ER<sup>+</sup>) breast cancer.

**Patients and Methods:** Paired gene expression and Ki67 protein expression were available from 69 postmenopausal women with ER<sup>+</sup> early breast cancer, at baseline and 2 weeks post-anastrozole treatment, in the presurgical setting. Functional gene modules ( $n = 26$ ) were retrieved from published studies and their module scores were computed before and after elimination of proliferation-associated genes (PAG). Ki67 and module scores were assessed at baseline and 2 weeks post-anastrozole. Unsupervised clustering was used to assess associations between modules and Ki67.

**Results:** Proliferation-based modules were highly correlated with Ki67 expression both pretreatment and on-treatment. At baseline with and without PAGs, Ki67 expression was significantly inversely correlated with ERG, ESR1.2, SET, and PIK3CA modules. Modules measuring estrogen signaling strongly predicted antiproliferative response to therapy with and without PAGs. Baseline expression of insulin-like growth factor-1 (IGF-I) module predicted a poor change in Ki67-implicating genes within the module as involved in *de novo* resistance to AIs. High expression of Immune.2.STAT1 module pretreatment predicted poor antiproliferative response to therapy. A significant association between estrogen-regulated genes modules (ESR1, ESR1-2, SET, and ERG) was evident post AI.

**Conclusions:** Multiple processes and pathways are affected by AI treatment in ER<sup>+</sup> breast cancer. Modules closely associated with ESR1 expression were predictive of good antiproliferative response to AIs, but modules representing immune activity and IGF-I/MAPK were predictive of poor Ki67 response, supporting their therapeutic targeting in combination with AIs. *Clin Cancer Res*; 20(9); 2485–94. ©2014 AACR.

### Introduction

About 75% to 80% of patients with breast cancer at primary diagnosis present with breast tumors that are hormone receptor positive disease (1). The standard of care for these patients is endocrine treatment, such as tamoxifen, which competes with estrogen for the estrogen receptor (ER), or aromatase inhibitors (AI) that block the conversion

of androgen to estrogen. The clinical efficacy of AIs is superior to that of tamoxifen for the treatment of postmenopausal women with ER-positive (ER<sup>+</sup>) breast cancer in both the adjuvant and metastatic setting (2). Neoadjuvant endocrine therapies, in particular AIs, are also effective at downstaging breast tumors and facilitating breast conservation in many postmenopausal patients (3).

Gene expression profiling has revealed the high degree of heterogeneity in breast cancer and the impact that particular molecular attributes can have on clinical outcome (4, 5). In particular, analyses of global gene expression profiles have revealed that breast cancer may be characterized as a collection of biologically distinct subgroups defined by "intrinsic" molecular markers. The principal subgroups include (i) luminal cancers, which are mainly ER $\alpha$ <sup>+</sup>, (ii) tumors characterized by amplification/overexpression of the *HER2* gene, and (iii) basal-like cancers in which hormone receptors and *HER2* are generally absent but basal cytokeratins are expressed. In addition to subtype-associated differences in prognosis and response to treatment, molecular differences within subtypes are increasingly being considered as

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### Translational Relevance

Response and resistance to endocrine therapy in estrogen receptor-positive (ER<sup>+</sup>) breast cancer is poorly understood. Using gene expression and Ki67 data from patients treated with the aromatase inhibitor (AI) anastrozole in the presurgical setting, we interrogated key biologic processes represented by gene modules ( $n = 26$ ). Proliferation (CIN70, GGI, AURKA, and Gene70) and estrogen-responsive pathways (ESR1.1, ESR1.2, and SET) were profoundly reduced by anastrozole treatment, as were signaling pathways PTEN, CASP3, E2F3, MYC, AKT/mTOR, and IGF-I. In contrast, PIK3CA, MAPK, Stroma.2-PLAU, Stroma.1, and Immune.1 expression module scores were significantly increased by treatment, suggesting that the pathways relevant to resistance are upregulated at this early time point. A significant association between estrogen-regulated modules (ESR1, ESR1-2, SET, and ERG) was evident post AI and may be indicators of late relapse. These observations reveal that different processes and pathways are affected differently with AI treatment between patients with ER<sup>+</sup> breast cancer, emphasizing the importance of tumor heterogeneity.

contributors to the diversity of treatment response. The luminal group is generally divided into two subgroups: luminal A tumors characterized by greater expression of estrogen-regulated genes and markers associated with luminal breast epithelial cells; and luminal B in which these features are less apparent and there is greater expression of proliferation-associated genes (PAG).

Despite the efficacy of endocrine therapy in patients with ER<sup>+</sup> breast cancer, approximately 25% of patients recur within 10 years after receiving 5 years of tamoxifen (6). Although the molecular events underpinning the efficacy of endocrine therapies are not fully understood (7, 8), it is clear that downregulation of proliferation plays a major role. Uncontrolled proliferation is a hallmark feature of tumor progression and in breast cancer this is most frequently assessed by immunohistochemical assessment of Ki67 (9). This marker has been used to assess prognosis, predict sensitivity or resistance to endocrine therapy, and serve as a dynamic biomarker of treatment efficacy in samples taken before, during, and after neoadjuvant endocrine therapy (10). Ki67 is suppressed to a variable extent by AIs in the large majority of breast cancer and overall by a mean of approximately 75% after 2 weeks, increasing to >80% after 12 to 16 weeks (11); the degree of Ki67 suppression by endocrine agents has been found to predict their efficacy as adjuvant therapy. Despite this focus of investigation on proliferation, it is known that many genes not associated with proliferation are also affected by estrogen deprivation (12). It seems likely that these other effects may contribute to the overall impact of AIs on clinical outcome. Preoperative neoadjuvant therapy provides a unique setting for characterizing such biologic changes in tumors and the molecular features that affect the changes.

To summarize the activity of biologic processes/pathways relevant to cancer, a number of gene modules have been derived, including those that represent Wnt, SRC, Ras, Myc, E2F3, PTEN loss (13, 14), activation of insulin-like growth factor 1 (IGF-I; ref. 15), mitogen-activated protein kinase (MAPK; ref. 16), AKT/mTOR pathways (17), and PIK3CA mutations (18). Key biologic processes in breast cancer have also formed the basis of functional gene signatures (19), including tumor invasion (Stroma-PLAU), immune response (Immune-STAT1), angiogenesis (VEGF), apoptosis (CASP3), proliferation (AURKA), ER signaling (ESR1), and HER2 signaling (ERBB2). Prognostic gene modules such as genomic grade index (GGI; ref. 5), Gene70 (20), and a signature that has been reported to recapitulate deficiency in DNA-repair mechanisms and chromosomal instability (CIN70; ref. 21) have also been described. These gene modules provide an opportunity to characterize the functional heterogeneity of ER<sup>+</sup> tumors and to study the potential impact of AI therapy on key biologic processes that may contribute to clinical benefit.

The primary objective of this study was to determine systematically whether baseline expression of 26 published gene modules predicted for antiproliferative response in ER<sup>+</sup> tumors with matched global gene expression data and Ki67 measurements, before and after 2 weeks of neoadjuvant anastrozole. Given that many of these gene modules contain PAGs, we first assessed the specific contribution of the PAGs to the correlations of the gene signatures with changes in Ki67. Second, we correlated the pretreatment expression of the individual gene modules with the change in Ki67 after 2 weeks of anastrozole therapy. Third, the effect of AI treatment on the gene modules was assessed and finally, the associations of the gene modules with Ki67 were determined before and after treatment, as well as their changes resulting from treatment.

## Materials and Methods

### Presurgical study of anastrozole

Pre- and on-treatment (week 2) core biopsies were available from postmenopausal women recruited into the anastrozole (1 mg/day)-only arm of a multicenter, randomized, double-blind, phase II neoadjuvant trial of anastrozole alone or with gefitinib in early breast cancer (ClinicalTrials.gov identifier: NCT00255463). This anastrozole-treated subgroup constitutes the Functional Aromatase Inhibitor Molecular Study (FAIMoS). Sample acquisition, storage, and RNA extraction was done as previously described (12). Following exclusions, paired samples were available from 81 patients treated for 2 weeks, of which 69 patients had corresponding Ki67 protein expression pre- and post treatment. A detailed CONSORT diagram is shown in Supplementary Fig. S1. These patients were used in the following analyses. Patient demographics are shown in Supplementary Table S2.

### Gene expression data and probe annotation

Gene expression profiles were generated on the Illumina HumanWG-6 v2 Expression BeadChip. Illumina raw data

were extracted using the BeadStudio software and were transformed and normalized using variance-stabilizing transformation and robust spline normalization method in the Lumi package in R (<http://www.bioconductor.org>). Probes were discarded if they were not detected in any of the 69 paired samples (detection  $P > 0.01$ ), resulting in 53.7% (26,163/48,701) of the probes remaining for the downstream data analysis. A corresponding annotation file, HumanWG-6\_V2\_0\_R4\_11223189\_A, was retrieved from the manufacturer's website (<http://www.switchto.com/annotationfiles.ilmn>). Gene expression data from this study were deposited by the authors of reference (12) and are available on <http://synapse.sagebase.org/#Dataset:16243> (22).

### Gene modules

Twenty-six publicly available gene modules representing potentially important biologic processes in breast cancer were assessed: (i) ER signaling and estrogen-regulated modules: ESR1.1, ESR1.2, sensitivity to endocrine therapy index (SET; refs. 19, 23, 24), and ERG (12); (ii) growth factor receptors and cytoplasmic signaling-related modules: IGF-1, obesity, MAPK (15, 16, 25), SRC, betacatenin, RAS (13), ERBB2, VEGF (19), PIK3CA (PIK3CA-GS; ref. 18), PTEN loss (14), and AKT/mTOR (17); (iii) cell cycle-related module: MYC (13); (iv) apoptosis and cell survival signaling related module: CASP3 (19); (v) proliferation-based modules: CIN70 (21), GGI (5), AURKA (19), Gene70 (20), and E2F3 (13); (vi) tumor invasion: Stroma.1 (26) and Stroma.2-PLAU (Stroma.2; ref. 19); (vii) immune response: Immune.1 (27) and Immune.2-STAT1 (Immune.2; ref. 19).

### Composition and mapping of the gene modules

EntrezGeneIDs were used as gene identifiers in all selected modules with the exception of the ESR1.1 module (23), in which the UniGeneID was used. In this instance, we converted to EntrezGeneID using the ROCK database ([www.rock.icr.ac.uk](http://www.rock.icr.ac.uk); ref. 28). The HumanWG-6\_V2 annotation file was used to map the EntrezGeneIDs to the corresponding Illumina probe IDs. When multiple probes mapped to the same EntrezGeneID, the probe with the highest variance across pre- and posttreatment samples was selected to represent the gene. Genes were discarded from further analysis if they were not mapped to either the annotation file or the expression data.

### PAGs

As Ki67 was used as the comparator with each of the gene modules, we hypothesized that PAGs, that occur in and dominate the behavior of many gene modules, may influence the representation of the key features in the respective process. To address this, three gene sets were identified, which were associated with proliferation: (i) genes functionally associated with cell-cycle progression and cell division according to Gene Ontology annotations (29), (ii) cycling genes (genes showing cell cycle stage-specific expression; ref. 29, 30), and (iii) tumor-based "proliferation cluster" genes (belonging to a 'proliferation cluster' defined

in human breast cancer expression datasets; refs. 19, 29, 31). This process identified a total of 1,178 PAGs (Supplementary Table S3).

### Computation of module scores

For each sample, a gene module score was calculated according to the expression of the relevant genes in the FAIMoS sample, as previously described (19, 32, 33).

$$\text{Module score}(s) = \sum_{i=1}^n W_i X_i / \sum |W_i|,$$

where  $n$  is the number of genes in a module,  $X_i$  represents the normalized gene expression in the test sample from the FAIMoS study, and gene-specific weights  $W_i$  are equal to +1 or -1 according to the direction of their association with the phenotype in the original publication. Data retrieved from publicly available datasets and the gene-set lists used in the study are detailed in Supplementary Table S4.

### Data analysis

All analyses were performed using R version 2.14.1 (<http://www.r-project.org/>).  $P$  values less than 0.05 were considered statistically significant. Reported  $P$  values are two-sided. Unadjusted  $P$  values are shown but because large numbers of statistical analyses have been performed, we have additionally shown adjusted  $P$  values to aid in their cautious interpretation by using R function `p.adjust.methods` (i.e., False discovery rate, FDR).

The effect of the 2-week AI treatment on  $\log_2$  Ki67 and gene modules was tested using Wilcoxon signed rank test based on the changes in expression. Geometric mean of intensity (G) was calculated as:

$$\log_2 G = \frac{1}{n} \sum_{i=1}^n \log_2 X_i$$

and G is the anti of  $\log_2^G$ , where  $X_i$  represents the gene expression intensity or Ki67 protein level. The % $\Delta$  of geometric mean of intensity in a module and in Ki67 was defined as

$$\% \Delta = \frac{(G_{\text{post}} - G_{\text{pre}})}{G_{\text{pre}}} \times 100$$

To study the associations of Ki67 and the twenty-six gene modules together with the impact of removing the PAGs, we performed (i) Spearman rank correlation between Ki67 and gene modules and (ii) `pvclust` (34) to visualize the degree of association between Ki67 and gene modules.

Both approaches were performed using  $\log_2$  Ki67 protein level and the expression module scores (i) at the baseline, (ii) in their response to estrogen deprivation, and (iii) posttreatment with and without PAGs. Here,  $\log_2^{(\text{Ki67pre})}$  and  $\log_2^{(\text{Ki67post})}$  were used for Ki67 pretreatment and posttreatment protein level respectively, and the 2-week protein level change in Ki67 was defined as  $\Delta \log_2^{\text{Ki67}} \left( \log_2^{(\text{Ki67post})} - \log_2^{(\text{Ki67pre})} \right)$ .



The R package pvclust, which calculates  $P$  values for each cluster using bootstrap resampling was used to assess the stability of the clusters. Two types of  $P$  values are available from this package: approximately unbiased (AU)  $P$  value and bootstrap probability (BP) value. The AU  $P$  value uses the multiscale bootstrap resampling methodology for calculating its  $P$  value and is superior to the BP  $P$  value calculated by the ordinary bootstrap resampling method. Hence, the AU method was used for these analyses and  $P$  values were reported as percentage (%), cluster with  $P$  value greater than 95% representing significantly associated modules.

To test the presence of clusters, we used the Ward method and correlation-based dissimilarity matrix with the parameters set to 10,000 bootstrap replicates, and the relative sample sizes set from 0.5 to 1.4 (default setting), with incremental steps of 0.1 to determine AU  $P$  value. In the data matrices, rows correspond to patients/samples, columns correspond to  $\log_2^{\text{Ki67}}$ /gene module scores, and the values were centered and scaled via R function scale.

## Results

### Effect of removal of PAGs on gene modules

As proliferation has been strongly associated with the predictive and prognostic utility of gene modules (35), we took the strategy of analyzing the endocrine responsiveness of the gene modules with and without PAGs (with the exception of the AURKA module given that its primary purpose was to represent proliferation; Supplementary Table S5). Analysis of the PAGs showed that they not only changed significantly with AI, but their change was significantly concordant with changes in Ki67 (Supplementary Table S6).

### Pretreatment associations of gene modules and Ki67 with and without the PAGs

Associations between the gene modules and Ki67 at baseline were assessed using pvclust. GGI, which mainly quantifies tumor proliferation, was in a significantly correlated cluster with AURKA, CIN70, PTEN loss, and Gene70 modules (AU  $P = 98\%$ ; Fig. 1A). Removal of the PAGs from these five gene modules resulted in the loss of the significance of the association with Gene70 and PTEN (Fig. 1B). Gene modules measuring estrogen signaling (ESR1.1, ESR1.2, and SET) and obesity with IGF-I remained significantly correlated in the absence or presence of PAGs. Another notable significant clustering was between AKT/mTOR, ERBB2, and VEGF, but this was disrupted by removal of the PAGs. In general, functionally related biologic processes were in highly correlated clusters.

At baseline, with and without PAGs, baseline Ki67 protein expression was significantly positively correlated with the following modules: GGI, CIN70, AURKA, PTEN, Gene70, IGF-I, obesity, ERBB2, and RAS, and inversely correlated with ERG, ESR1.2, SET, and PIK3CA gene modules (Supplementary Table S7A and S7B). The significance of these associations with the proliferation-based modules, GGI, CIN70, AURKA, PTEN, and Gene70, became weaker

after eliminating PAGs, whereas the relationship of Ki67 with the remaining modules, IGF-I, obesity, ERBB2, RAS, ERG, ESR1.2, SET, and PIK3CA was unaffected.

### Correlation of pretreatment gene modules with change in Ki67

To assess the effect of the gene modules at predicting antiproliferative response to AI therapy, we compared baseline expression of each gene module versus the change ( $\Delta$ ) in Ki67 as a marker of response (Table 1), in which a negative  $\rho$  indicates a positive relationship of the module or metagene with Ki67 response to AI therapy, because the response is a reduction in Ki67. We found that the ER signaling module scores (ESR1.1, SET, and ESR1.2) were significantly associated with the reduction in Ki67 (unadjusted  $P < 0.005$ , 0.04, and  $< 0.05$ , respectively). In contrast, Immune.2, STAT1, Gene70, and IGF-I baseline module scores were significantly negatively associated with change in Ki67, indicating that the higher the module score, the smaller the antiproliferative response. This inverse correlation became stronger when PAGs were removed from the Gene70 and GGI modules. The ESR1.1 and Immune.2, STAT1 modules remained significant (adjusted  $P < 0.05$ ) after adjustment for multiple testing.  $\rho$  values for the relationship of obesity, Stroma.2, PLAU and MAPK modules with change in Ki67 were all positive, but with  $0.05 < \text{unadjusted } P < 0.10$ .

### Dynamic expression changes in gene modules and Ki67 in response to 2 weeks of AI treatment

Fourteen gene module scores (including PAGs) were significantly decreased after 2 weeks of anastrozole therapy, although none to the same magnitude as the single immunohistochemistry marker Ki67 (Table 2). The ERG module showed the most significant change in response to therapy. CIN70, GGI, AURKA, and the Gene70 module, which mainly quantify tumor proliferation, were each profoundly reduced by anastrozole. For CIN70, GGI, AURKA, and ERG, the  $P$  values for the change were all relatively similar to that for Ki67 at  $c.10^{-12}$ . Modules representing SET, PTEN, CASP3, E2F3, MYC, ESR1.1, ESR1.2, AKT/mTOR, and IGF-I were also all significantly suppressed but at a lesser magnitude by estrogen deprivation. In contrast, MAPK, Stroma.2, PLAU, Stroma.1, and Immune.1 expression module scores were significantly increased by anastrozole treatment. When the PAGs were removed from the gene modules, the change in expression was significantly reduced in each of the proliferation-based modules. In particular, Gene70 was profoundly affected and was no longer significantly suppressed by AI treatment. However, removal of the PAGs from the remaining gene modules namely ERG, ESR1.1, ESR1.2, SET, MYC, MAPK, Stroma.2, PLAU, and Stroma1 had no significant impact.

### Associations of changes in gene modules and changes in Ki67 with and without PAGs

Assessment of the interconnectivity of the changes in the modules during treatment was strikingly different. ERBB2

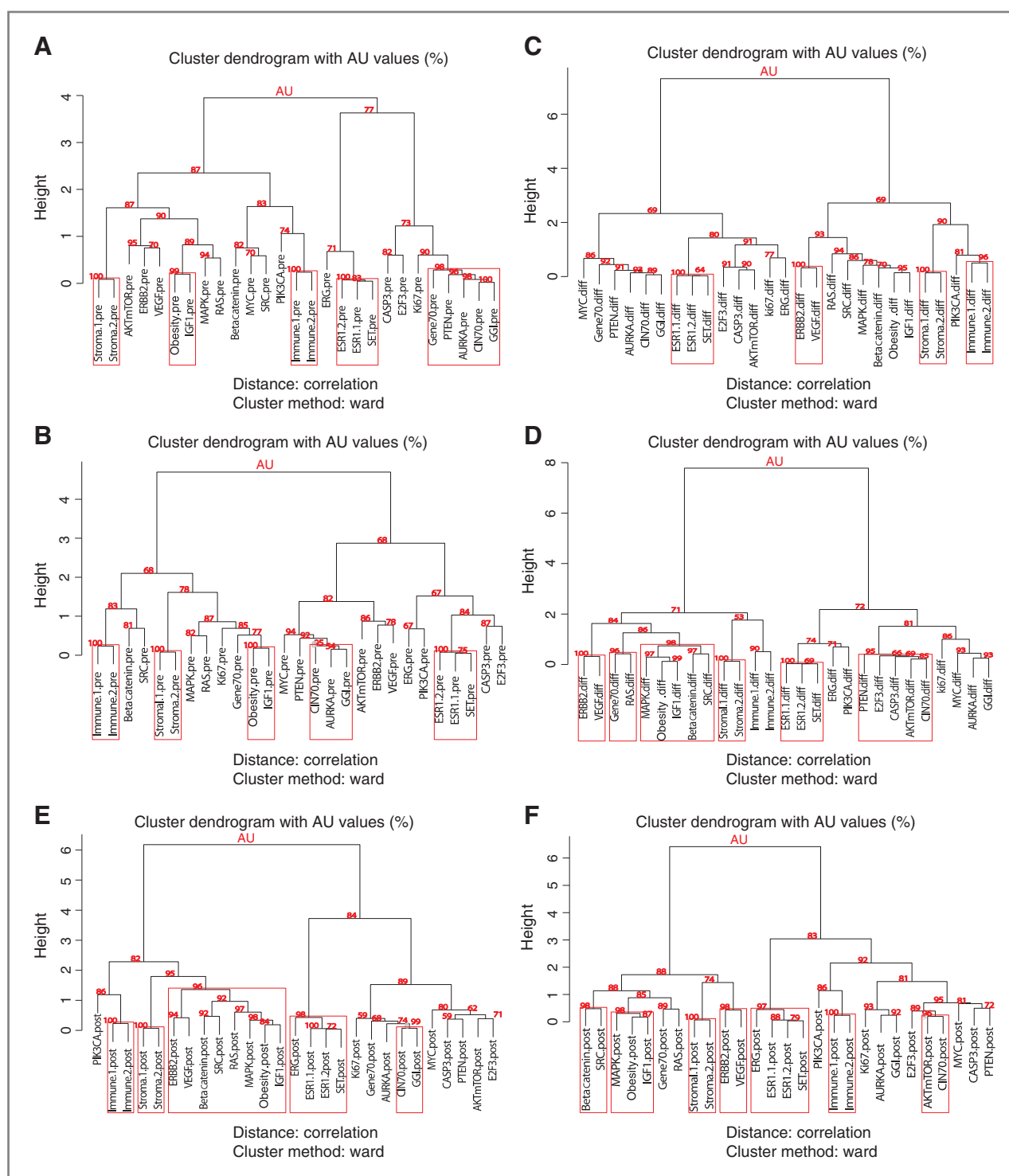


Figure 1. Cluster dendrogram pretreatment includes PAGs (A), excluding PAGs (B); cluster dendrogram of changes in expression after treatment includes PAGs (C), excluding PAGs (D); cluster dendrogram of posttreatment includes PAGs (E), excluding PAGs (F). The cluster stability analysis of the unsupervised hierarchical clustering uses pvclust to calculate the AUP value. The AUP values are reported as percentage (%) in red. Clusters representing significantly associated modules (i.e., those clusters with greater than 95% significance) are shown by the red rectangle. Gene module scores and Ki67 protein expression at pretreatment, posttreatment, and change in the treatment were used for this analysis.

with VEGF, Strom.1 with Strom.2.PLAU, and ESR1.1 with ESR1.2 and SET remained in their significantly associated cluster irrespective of the presence of PAGs (Fig. 1C & D).

However, removal of the PAGs resulted in a strong association between IGF-I, obesity, MAPK, Src, and betacatenin, also, between changes in PTEN loss, E2F3, CASP3, AKT/

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**Table 1.** Spearman correlation between individual pretreatment module score and change in Ki67 protein expression,  $\Delta \log_2^{Ki67} \left( \log_2^{(Ki67_{post})} - \log_2^{(Ki67_{pre})} \right)$ 

Module name	All matched			PAGs excluded			Reference
	$\rho$	Unadjusted <i>P</i>	Adjusted <i>P</i>	$\rho$	Unadjusted <i>P</i>	Adjusted <i>P</i>	
<b>ESR1.1</b>	<b>-0.337</b>	<b>0.0046</b>	<b>0.0469</b>	<b>-0.339</b>	<b>0.0044</b>	<b>0.0469</b>	Mackay et al. (23)
<b>SET</b>	<b>-0.248</b>	<b>0.0396</b>	0.1803	<b>-0.252</b>	<b>0.0370</b>	0.1803	Symmans et al. (24)
<b>ESR1.2</b>	<b>-0.241</b>	<b>0.0465</b>	0.1803	<b>-0.244</b>	<b>0.0431</b>	0.1803	Desmedt et al. (19)
ERG	-0.226	0.0619	0.1860	-0.206	0.0890	0.2188	Dunbier et al. (12)
MYC	-0.160	0.1889	0.3705	-0.147	0.2282	0.4157	Bild et al. (13)
VEGF	-0.065	0.5955	0.7820	-0.065	0.5955	0.7820	Desmedt et al. (19)
E2F3	-0.041	0.7374	0.8646	-0.052	0.6729	0.8579	Bild et al. (13)
Betacatenin	0.015	0.9016	0.9384	-0.002	0.9888	0.9888	Bild et al. (13)
AKT/mTOR	0.026	0.8294	0.9000	-0.006	0.9587	0.9779	Majumder et al. (17)
ERBB2	0.042	0.7313	0.8646	0.037	0.7629	0.8646	Desmedt et al. (19)
SRC	0.048	0.6942	0.8635	0.033	0.7890	0.8748	Bild et al. (13)
PIK3CA	0.065	0.5980	0.7820	0.071	0.5638	0.7820	Loi et al. (18)
PTEN	0.072	0.5545	0.7820	0.038	0.7536	0.8646	Saal et al. (14)
Stroma.1	0.122	0.3196	0.5046	0.127	0.2991	0.4921	Farmer et al. (26)
CASP3	0.127	0.2971	0.4921	0.092	0.4520	0.6780	Desmedt et al. (19)
RAS	0.132	0.2808	0.4921	0.120	0.3265	0.5046	Bild et al. (13)
CIN70	0.152	0.2123	0.4010	0.017	0.8891	0.9384	Carter et al. (21)
Immune.1	0.161	0.1866	0.3705	0.161	0.1866	0.3705	Teschendorff et al. (27)
AURKA	0.175	0.1507	0.3342	N/A	N/A	N/A	Desmedt et al. (19)
Obesity	0.206	0.0901	0.2188	0.196	0.1064	0.2467	Creighton et al.
GGI	0.217	0.0731	0.1962	<b>0.277</b>	<b>0.0213</b>	0.1552	Sotiriou et al. (5)
Stroma.2.PLAU	0.221	0.0679	0.1924	0.226	0.0620	0.1860	Desmedt et al. (19)
MAPK	0.230	0.0576	0.1860	0.237	0.0495	0.1803	Creighton et al. (16)
<b>IGF-I</b>	<b>0.250</b>	<b>0.0385</b>	0.1803	<b>0.244</b>	<b>0.0433</b>	0.1803	Creighton et al. (15)
<b>Gene70</b>	<b>0.299</b>	<b>0.0127</b>	0.1080	<b>0.357</b>	<b>0.0026</b>	0.0442	van't Veer et al. (20)
<b>Immune.2.STAT1</b>	<b>0.381</b>	<b>0.0013</b>	<b>0.0332</b>	<b>0.381</b>	<b>0.0013</b>	<b>0.0332</b>	Desmedt et al. (19)

NOTE: The bolded letters and figures represent the gene modules that are significantly altered.

mTOR, and CIN70. Notably, this was not a result of shared genes between the modules (Supplementary Table S8).

The change in expression of the ERG module was significantly correlated with the change in Ki67 protein level. Similar correlations with Ki67 were evident for the modules CIN70, GGI, AURKA, AKT/mTOR, PTEN, Gene70, and E2F3. However, these associations became weaker or even non-existent after removal of the PAGs from these modules, indicating that the previous correlations were partially dependent on the colinearity provided by the PAGs within those modules (Supplementary Table S9A and S9B).

#### Posttreatment associations of gene modules and Ki67 with and without the PAGs

After 2 weeks of treatment, the gene modules were clustered mainly into five pathway-associated clusters as follows (Fig. 1E & F): (i) ER signaling cluster consisting of ESR1-1, ESR1-2, SET, and ERG was evident ( $AU P \geq 97\%$ ); of note, these modules shared only two genes (NAT1, TFF1); (ii) cell proliferation related cluster; (iii) growth factor receptors and cytoplasmic signaling-related cluster, which contained IGF-I, obesity, MAPK, RAS, ERBB2, VEGF, betacatenin, and

SRC gene modules; (iv) tumor invasion cluster (Strom.1, Strom.2.PLAU); and (v) a significantly correlated immune response cluster (Immune.1, Immune.2.STAT1).

Interestingly, in these on-treatment samples, removal of the PAGs had no significant impact on the clustering. This is likely due to the suppression of proliferation in most samples by the AI. Of note, with and without PAGs, the PIK3CA module was not highly associated with any of the clusters, despite its module score being significantly positively correlated with ERG module score (unadjusted  $P = 0.007$  and  $0.004$  respectively), weak but significantly inversely correlated with obesity module score (unadjusted  $P = 0.013$  and  $0.024$ , respectively; Supplementary Table S10A and S10B).

Posttreatment Ki67 values were significantly positively associated with the proliferation-based modules (Gene70, AURKA, CIN70, and GGI), PTEN, IGF-I, AKT/mTOR, RAS, and Immune.2.STAT1 with and without PAGs, despite the decreased proliferation. However, weak but significant negative correlations were evident between 2-week Ki67 values with expression module scores ESR1.1 (with and without PAGs, unadjusted  $P = 0.027$

**Table 2.** Dynamic changes in expression of Gene modules and Ki67 in response to the 2 weeks' AI treatment

Module name	All matched			PAGs excluded		
	% $\Delta^a$ of Geometric mean of intensities of a module of pre- and posttreatment	Two-sided unadjusted <i>P</i> value of Wilcoxon test	Adjusted <i>P</i>	% $\Delta$ of Geometric mean of intensities of pre- and posttreatment	Two-sided unadjusted <i>P</i> value of Wilcoxon test	Adjusted <i>P</i>
Ki67	<b>-75.23</b>	<b>4.75E-12</b>	<b>4.77E-11</b>	N/A	N/A	N/A
ERG	<b>-28.36</b>	<b>1.40E-12</b>	<b>3.64E-11</b>	<b>-28.51</b>	<b>1.20E-12</b>	<b>3.64E-11</b>
CIN70	<b>-24.25</b>	<b>4.20E-12</b>	<b>4.77E-11</b>	<b>-11.34</b>	<b>1.60E-08</b>	<b>7.56E-08</b>
GGI	<b>-22.32</b>	<b>5.00E-12</b>	<b>4.77E-11</b>	<b>-7.82</b>	<b>3.90E-09</b>	<b>2.54E-08</b>
AURKA	<b>-12.34</b>	<b>5.50E-12</b>	<b>4.77E-11</b>	N/A	N/A	N/A
Gene70	<b>-8.37</b>	<b>8.60E-09</b>	<b>4.47E-08</b>	-1.11	0.3400	0.3930
SET	<b>-7.99</b>	<b>1.00E-05</b>	<b>3.25E-05</b>	<b>-8.20</b>	<b>6.70E-06</b>	<b>2.32E-05</b>
PTEN	<b>-7.22</b>	<b>2.80E-10</b>	<b>2.08E-09</b>	<b>-2.61</b>	<b>0.0002</b>	<b>3.85E-04</b>
CASP3	<b>-5.19</b>	<b>0.0114</b>	<b>0.0180</b>	<b>-4.45</b>	<b>0.0420</b>	<b>0.0642</b>
E2F3	<b>-4.40</b>	<b>4.40E-09</b>	<b>2.54E-08</b>	<b>-2.63</b>	<b>8.10E-05</b>	<b>1.91E-04</b>
MYC	<b>-4.32</b>	<b>1.80E-06</b>	<b>7.20E-06</b>	<b>-4.74</b>	<b>3.90E-07</b>	<b>1.69E-06</b>
ESR1.2	<b>-4.39</b>	<b>2.00E-04</b>	<b>3.85E-04</b>	<b>-4.53</b>	<b>2.00E-04</b>	<b>3.85E-04</b>
ESR1.1	<b>-4.18</b>	<b>0.0015</b>	<b>0.0027</b>	<b>-4.56</b>	<b>5.00E-04</b>	<b>9.29E-04</b>
AKT/mTOR	<b>-2.75</b>	<b>0.0052</b>	<b>0.0085</b>	-2.07	0.0590	0.0877
IGF-I	<b>-2.44</b>	<b>3.80E-05</b>	<b>9.88E-05</b>	<b>-1.94</b>	<b>2.00E-04</b>	<b>3.85E-04</b>
VEGF	-1.80	0.1300	1.73E-01	-1.80	0.1300	0.1730
RAS	-1.14	0.1100	1.55E-01	-1.00	0.2500	0.3020
SRC	0.52	1.0000	1.0000	0.34	0.7700	0.7850
PIK3CA	0.47	0.3400	0.3930	0.28	0.4700	0.5200
Obesity	0.74	0.7100	0.7380	0.67	0.6100	0.6470
Betacatenin	1.01	0.3800	0.4300	0.73	0.4900	0.5310
ERBB2	1.62	0.1700	0.2100	2.12	0.0770	0.1110
Immune.2.STAT1	3.62	0.1400	0.1780	3.69	0.1400	0.1780
MAPK	<b>5.03</b>	<b>4.80E-06</b>	<b>1.78E-05</b>	<b>4.50</b>	<b>2.20E-05</b>	<b>6.73E-05</b>
Stroma.2.PLAU	<b>7.57</b>	<b>0.0038</b>	<b>0.0066</b>	<b>7.72</b>	<b>0.0040</b>	<b>0.0067</b>
Immune.1	<b>13.45</b>	<b>3.20E-05</b>	<b>8.76E-05</b>	<b>13.45</b>	<b>3.20E-05</b>	<b>8.76E-05</b>
Stroma.1	<b>20.75</b>	<b>4.60E-05</b>	<b>1.14E-04</b>	<b>20.05</b>	<b>8.60E-05</b>	<b>1.94E-04</b>

NOTE: The bolded letters and figures represent the gene modules that are significantly altered.

<sup>a</sup>% $\Delta$  of Geometric mean of intensities of pre and post treatment = [(post-geometric mean intensity - pre-geometric mean intensity)/pre-geometric mean intensity]  $\times$  100.

and 0.036, respectively). Two-week Ki67 was significantly positively associated with CASP3 and E2F3 only when PAGs were present in modules (unadjusted *P* = 0.014 and 0.025, respectively), and the statistical significance was lost in both cases when PAGs were removed from the modules (unadjusted *P* = 0.062 and 0.317, respectively; Supplementary Table S10A and S10B).

## Discussion

The goal to predict those patients most likely to gain benefit from or be resistant to endocrine therapy remains a major clinical challenge. Investigations in the neoadjuvant setting are favored for study of mechanisms. In the current study, we used the change in Ki67 as the index of response. Although pathologic complete response (pCR) is a validated endpoint for chemotherapy in ER<sup>-</sup> or HER2<sup>+</sup> disease, it

is not a valuable endpoint in ER<sup>+</sup> disease, whether this is treated with endocrine therapy or chemotherapy (36, 37). Consistent with this, in the clinical trial from which the data discussed were derived, no patient showed pCR on any arm of the trial (11). In contrast, the change in Ki67 is a widely accepted biomarker of endocrine treatment efficacy (9, 38).

Over the last decade, a vast amount of gene expression data has been generated and used to develop gene modules or metagenes that represent numerous individual pathways or processes. Some are either prognostic or predictive of response to therapy. In the current study, for the first time, we assessed the ability of 26 publicly available gene expression modules encompassing a number of processes associated with tumor biology, to predict Ki67 response to an AI. We also assessed the impact of AI treatment on their expression.



As expected, gene modules based around proliferation (GGI, Gene70, CIN70, and AURKA) correlated strongly with the proliferation marker Ki67. The differences in the correlation of many of the modules with one another, when PAGs were removed, emphasize the influence of proliferation features in many of them and also the importance of their removal, to consider the biologic importance of the modules and represented processes in relation to highly antiproliferative agents, such as AIs. The observation that removal of PAGs had little impact on clustering in the on-treatment samples in which proliferation was markedly reduced suggests the effectiveness of this subtractive approach.

At baseline, expression of the ERG module, consisting of estrogen-regulated genes, showed a trend toward predicting a change in Ki67 as a marker of response to therapy. In addition, the ERG module scores were the most significantly reduced after the 2 weeks' AI treatment. This latter observation supports the constituent genes of the ERG module as being highly estrogen dependent and their baseline expression, as somewhat indicative of endocrine responsiveness. However, it should be noted that this gene signature was originally derived from this cohort of patients (8).

The ESR1.1, ESR1.2, and the SET modules were generated using an association-based approach in which all genes highly correlated with and including ESR1 expression were included (19, 23, 24). In this study, we found that high baseline module scores of these gene signatures predicted good Ki67 response to AI therapy more strongly than the ERG module, indicating that ER-associated rather than estrogen-regulated genes are more important for predicting response to AIs.

The IGF, together with its receptor (IGF-IR), has been strongly implicated in endocrine resistance via hyperactivation of both the PI3K and MAPK pathways, leading to the ability of tumors to circumvent the need for steroid hormones (15, 39, 40). This is consistent with high IGF modules scores being associated with poor response to anastrozole. Furthermore, the MAPK module provided similar trend when PAGs were removed from the module. Not surprisingly, association studies between the gene modules showed that IGF-I was correlated with both the obesity and MAPK gene modules as well as with the obesity module itself, showing a trend to association with poor Ki67 response. This last observation is particularly notable given the recent evidence for obesity being associated with reduced benefit from AIs relative to that from tamoxifen (25).

The PIK3CA-GS (18) showed that at baseline the module was significantly inversely correlated with Ki67, IGF-I, MYC, E2F3, PTEN loss, and gene modules indicative of proliferation. This suggests the lower the PIK3CA-GS signature score the greater the pathway activity, but does not predict change in Ki67, consistent with our recent data on PIK3CA mutations, having no impact on Ki67 response in these same samples (41). One explanation for this observation is that the PIK3CA-GS was derived from a PIK3CA exon 20 mutation. As such, the gene signature may have greatest relevance in selecting patients most likely to respond to

PI3K/mTOR inhibition. A recent retrospective study (42) of patients receiving the mTOR inhibitor everolimus in combination with letrozole in the neoadjuvant setting showed, surprisingly, that PIK3CA mutations did not predict for response to the combination or either agent but the PIK3CA-GS was able to identify those patients with ER<sup>+</sup> breast cancer who gained benefit from the combination.

One of the most striking observations was that Immune.2.STAT1 predicted poor Ki67 response to anastrozole. This is consistent with our recent report showing that immune-related genes are highly predictive of poor antiproliferative response to AIs (12). This is further supported by the recently published article by Tsang and colleagues (43), which showed that higher lymphocytic infiltration correlated with poor prognosis in ER<sup>+</sup> patients. The data suggest that patients showing evidence of high immune-related gene expression and/or lymphocytic infiltration may be candidates for treatment with immune-modulators in combination with an AI. It is, however, in stark contrast with that seen for response to chemotherapy in which high expression of these immune modules was associated with a significantly better outcome in two independent studies (19, 27). A dynamic network of positive and negative regulatory cytokines, which influence not only the type of response but also the amplitude of that response, modulates the immune system. For instance, the immune system has conflicting roles in tumor progression, on the one hand suppressing tumor growth, whereas on the other, providing a proproliferative environment by generating a source of growth factor cues. Hence, one explanation for this result could be that chemotherapy destroys not only the tumor, but also immune-associated cells, whereas endocrine therapy impacts more on cross-talk between the tumor and the immune system. This hypothesis is supported to some extent by recent data, which suggest that letrozole can decrease recruitment of regulatory T cells, which are known to dampen the immune response (44).

Despite these distinct correlations between the E2F3 signature and other modules, no correlation was found between E2F3 and Ki67 protein expression at the baseline, in the presence or absence of the PAGs. This contrasts with a previous study (45), which suggested that an E2F3 module, consisting of 24 non-cell-cycle genes, was significantly associated with Ki67 protein expression at baseline. Furthermore, the authors suggested the expression signature of E2F3 activation in ER<sup>+</sup> tumors maybe linked with resistance to AI-induced estrogen deprivation. One potential explanation for these conflicting observations is that 13 of the 24 genes within the E2F3 signature (45) are known PAGs, accounting for the strong correlation with Ki67 protein levels (45).

The biology of breast cancer does not seem conducive to the identification of robust predictive signatures. The molecular mechanisms underpinning resistance to targeted therapeutics may be confined to the functional alteration of key genes, which may not necessarily influence global gene expression profiles. Another important caveat is that resistance mechanisms, such as gene mutations and epigenetic modifications, may be beyond the perception of gene



expression analysis, limiting the identification of potentially important predictive alterations (46–48). The heterogeneity of breast cancer also raises the possibility that resistance may emerge through selection of nonmodal subpopulations, which are unable to be discerned by the composite nature of microarray-based analyses (49, 50). Technological advances have the potential to address some of these shortfalls; in particular, the advent of next-generation sequencing technology provides the opportunity for DNA and RNA aberrations to be integrated with transcriptional profiling.

In conclusion, the molecular response to AI treatment varies greatly between patients, consistent with the variable clinical benefit, as a result of tumor heterogeneity. Multiple processes and pathways are affected by AI treatment in ER<sup>+</sup> breast cancer; however, reduced proliferation seems to be the dominant process. As expected, those modules most closely associated with ESR1 expression were predictive of good antiproliferative response to AIs. Of note, modules representing immune activity and IGF-1/MAPK were predictive of poor Ki67 response to therapy, supporting our earlier study (8) and highlighting their potential utility as biomarkers.

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M. Dowsett has received commercial research grants and speakers bureau honoraria from AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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