Gene Expression Signatures of BRCAness and Tumor Inflammation Define Subgroups of Early-Stage Hormone Receptor–Positive Breast Cancer Patients

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ABSTRACT

Purpose: Patients with estrogen receptor– and/or progesterone receptor–positive, early breast cancer benefit from hormonal treatment, yet high global death burdens due to high prevalence and long-term recurrence risk call for biomarkers to guide additional treatment approaches.

Experimental Design: From a prospective, observational study of postmenopausal early breast cancer patients treated with tamoxifen or aromatase inhibitors, gene expression analyses of 612 tumors was performed using the NanoString Breast Cancer 360 panel to interrogate 23 breast cancer pathways. Candidate signatures associated with disease subtype and event-free survival (EFS) were obtained by cluster analysis, Cox modeling, and conditional inference trees, and were independently tested in 613 patients from BreastMark. Tumor-infiltrating lymphocytes (TIL) were assessed on tissue sections, and mutational burden was assessed in 36 tumors by whole-exome sequencing.

Results: PAM50-derived classification distinguished lower-risk (Luminal A) from higher-risk subtypes (Luminal B, $P = 0.04$; HER2, $P = 0.006$; Basal, $P = 0.008$). In higher-risk patients, shorter EFS was associated with low androgen receptor [HR = 3.61; 95% confidence interval (CI), 1.72–7.56; $P = 0.001$] or high BRCAness signature expression (HR = 3.58; 95% CI, 1.19–10.7; $P = 0.023$). BRCAness was independently confirmed as a predictor of shorter EFS (HR = 2.64; 95% CI, 1.31–5.34; $P = 0.007$). About 13%–15% of patients, enriched for high-grade, higher-risk subtypes ($P \leq 0.0001$), had strong expression of the Tumor Inflammation Signature (TIS) suggestive of an inhibited antitumor immune response. TIS scores were strongly associated with TIL numbers ($P < 1e\text{-}30$) but not with tumor mutation status.

Conclusions: BRCA-related DNA repair deficiency and suppressed tumor immune responses may be clinically relevant predictors of endocrine therapy complementing treatment options in subgroups of hormone-sensitive early breast cancer.

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Introduction

Adjuvant endocrine therapy is the cornerstone of breast cancer treatment of patients with estrogen receptor (ER)- and/or progesterone receptor (PR)-positive early disease for the long-term estrogen deprivation to prevent breast cancer recurrence and death (1). Standard treatments include the selective estrogen receptor modulator (SERM) tamoxifen (TAM) that interferes with estrogen binding at the ER, and aromatase inhibitors (AI) that block the enzyme aromatase to prevent the conversion of androgens to estrogens. Five- to 10-year adjuvant endocrine therapy significantly reduces recurrence and mortality rates; however, recurrence and death events occur in about one third of treated patients due to the persistent long-term risk of therapy failure (2, 3).

Failure of adjuvant endocrine treatment is attributed to multiple biological conditions including metabolic resistance (changes in endogenous ligand or drug exposure; refs. 4–7), acquired resistance (change in drug response; refs. 8, 9), and intrinsic resistance (differential tumor cell biology; refs. 10, 11). Standard prognostic factors include increased hormone receptors ER and PR as well as growth factor receptor HER2 expression. Recent advances introduced genomic (mutational) signatures and multiple gene expression signatures that provided new insight into tumor cell heterogeneity and prognosis (12–16). Prominent transcriptome signatures used for breast cancer risk classification and decision making in favor of or against chemotherapy or an extended adjuvant endocrine treatment include the 21-gene recurrence score (Oncotype DX), Breast Cancer

Translational Relevance

Antihormonal agents are lowering recurrence and death rates in patients with estrogen receptor– or progesterone receptor–positive, early breast cancer, yet the prevention of treatment failure may require adapted treatment regimens for patients with higher-risk tumor characteristics. Our study unveils the presence of patient subgroups with an increased BRCAness score or high tumor inflammation signature (TIS) who may potentially benefit from additional therapies targeting BRCA-related DNA repair deficiency or immune checkpoints, respectively. Both markers can be easily identified in primary formalin-fixed, paraffin-embedded tumor specimen and therefore a biology-guided complementation of standard adjuvant therapy may be feasible. This opens the prospect to improve the clinical management of hormone receptor–positive early breast cancer, thereby reducing the burden of treatment failure in patients with aggressive tumors.

Index, EndoPredict, MammaPrint, and the PAM50-based Prosigna [risk of recurrence (ROR)] test (17–20). While these risk score assays show prognostic utility and have become indispensable for the guidance of systemic adjuvant treatment, their informative value is limited with regards to known available targeted therapies. Of those, PI3K/mTOR inhibitors, CDK4/6 inhibitors, DNA methylation agents, HER2 and RAF/MEK targeted therapy, PARP inhibitors, and DNA repair pathway agents, as well as immunotherapeutic agents (immune checkpoint inhibitors), have been shown efficacious in aggressive and advanced disease such as HER2-amplified, triple-negative (TNBC), and metastatic breast cancer. Potentially, this arsenal may be useful also in subsets of early breast cancer. In particular, patients with early-stage ER- and/or PR-positive breast cancer at increased risk of adjuvant treatment failure might benefit from such biology-guided targeted therapies via an upgrade or addition of their standard chemo-endocrine treatment, as exemplified by the use of anti-HER2 agents in HER2-amplified breast cancer. This view is encouraged, for example, by recent findings of single-cell proteome landscapes in high-grade ER-positive tumors showing high frequencies of PD-L1–positive tumor-associated macrophages and exhausted T cells indicative of an immunosuppressed stage (21). As the role of immune response mechanisms in luminal breast cancer is yet unclear (22, 23), similar findings in ER-positive patients confirming those reported by Wagner and colleagues (21) could provide a rationale for immune checkpoint blockade as an additional treatment option to enable more favorable outcomes in higher-risk patients, similar to that observed in patients with advanced TNBC (24, 25).

The nCounter Breast Cancer 360 (BC360; NanoString) 770-gene expression panel classifies breast cancers by virtue of 23 defined key pathways including transcriptional regulation, tumor microenvironment, growth and steroid-receptor signaling, as well as immune response. The panel also includes the previously validated PAM50 and derived ROR score, as well as Tumor Inflammation Signature (TIS) that detects a peripherally suppressed preexisting adaptive immune response identified in many different tumor types including TNBC (26). While the PAM50 classifier stratifies breast cancers into intrinsic subtypes of different prognosis including Luminal A (LumA), Luminal B (LumB), HER2-enriched (HER2), basal-like (Basal), and normal-like tumors, these subtypes can now be further scrutinized for their recurrence and death risk conferred by specific single or multiple deregulated pathways.

Our primary objective was to provide a basis for the discovery of novel biomarkers for the selection of available targeted treatments, and moreover, to provide insight into putative novel treatment targets to be adopted from tumor biology. Here we present novel biology-informed predictors from a BC360 expression screen of a large patient cohort with ER- and/or PR-positive early breast cancer with completed 5 years of endocrine treatment (TAM, AI, or switch therapy) that have been independently confirmed using the publicly available BreastMark transcriptome and clinical dataset (27).

Materials and Methods

Patient cohorts

Two independent cohorts of hormone receptor–positive, postmenopausal early-stage breast cancer patients were used which served for discovery (IKP211, $N = 612$) and verification (BreastMark, $N = 613$; see Supplementary Fig. S1 for CONSORT study flow diagram).

Discovery cohort

The gene expression and outcome association analysis were based on the clinical data and formalin-fixed paraffin-embedded (FFPE) tumor tissues of 612 patients that represent a subset of the IKP211 study (German clinical trials register DRKS00000605). The IKP211 study is a prospective multicenter observational breast cancer study for the purpose of investigating endocrine treatment efficacy and toxicity. Patients ($N = 1,286$) were recruited between 2005 and 2011 across 36 German breast centers and hospitals (see Appendix). Inclusion criteria were a first diagnosis of invasive early ER-positive and/or PR-positive breast cancer in postmenopausal women and 5-year intended endocrine treatment with either TAM or an AI as monotreatment, respectively, or switch treatment between TAM and AI in line with the current clinical practice following surgery with or without subsequent radiation-chemotherapy. From the 1,020 eligible patients with available tumor tissue, two thirds of patients (earliest enrolled) were selected. Tumor tissues with at least 20% tumor cells and evaluable for immune cell infiltrate were included ($n = 624$) and successfully profiled ($n = 612$). The study was carried out in accordance with the provisions of the Declaration of Helsinki of 1975. Informed written consent was obtained from each patient, and ethics approval was obtained from the Ethics Commission of the University of Tübingen (Tübingen, Germany), and respective local ethics committees of participating centers.

Independent verification cohort

Data from patients with breast cancer were retrieved from the publicly available BreastMark database that combines multiple breast cancer microarray gene expression datasets and related clinical outcome information for the analysis of up to 4,739 patients with breast cancer (27). The independent cohort ($N = 613$) was defined upon filtering patients for ER-positive status, postmenopausal status (inferred from age at diagnosis using >50 years as a cutoff when menopausal status was missing), available gene expression data (referring to 12,403 Entrez gene IDs), and pre-assigned PAM50 breast cancer subtypes LumA, LumB, HER2, and Basal. Among those, 504 patients had complete clinical follow-up data and were used for the confirmation of clinical associations (Supplementary Table S1). Endocrine treatment information was missing in 40% of patients, thus analyses were based on PAM50-defined risk groups.

Tumor processing

ER, PR, and HER2 tumor status in the IKP211 cohort were determined by IHC following standard procedures at the participating pathologic institutes. Hematoxylin and eosin staining was performed for each FFPE tumor block to verify the presence of at least 20% tumor cells in the respective sections. Tumor-infiltrating immune cells (TIL), mainly composed of T lymphocytes, plasma cells, and occasionally monocytes, were centrally assessed at the Department of Pathology of the Robert Bosch Hospital Stuttgart following standard guidelines (28). Briefly, tumor stroma was evaluated for the presence of TILs by quantitating the proportion of stroma area infiltrated by immune cells (0%–100%). Counts were rounded to the nearest 5% and averaged from multiple areas if tumors were heterogenous. For nucleic acid extraction, five 10-μm sections were cut from each tumor tissue block and stored at 4°C. RNA and DNA were extracted from 4–5 sections using a simultaneous DNA/RNA protocol (Quick-DNA/RNA FFPE, ZymoResearch), quantitated by spectrometry, and stock solutions were kept frozen at −80°C until use.

Gene expression analysis

A total of 250 ng total RNA was subjected to gene expression profiling using the nCounter FLEX Analysis System (NanoString Technologies). We utilized the NanoString BC360 panel comprising 770 genes (Supplementary Table S2) across 23 key breast cancer pathways/processes including the prognostic PAM50 signature for intrinsic subtype classification. Raw counts were quality controlled, background subtracted, and normalized using 18 housekeeping genes in nSolver Analysis Software (v4.0). Expression values were $\log_2$-transformed for statistical analysis. Tumors were classified into one of the four intrinsic subtypes, Luminal A (LumA), Luminal B (LumB), Basal-like (Basal), and HER2-Enriched (HER2), based on the PAM50 classifier algorithm (29). In total, 34 gene signatures including breast cancer key genes related to tumor immune signaling, tumor regulation, steroid receptor signaling, tumor microenvironment, hypoxia, and drug responsiveness were assessed. The TIS is a validated 18-gene classifier (Supplementary Table S3) that measures a preexisting peripherally suppressed adaptive immune response predicting response to immune checkpoint blockade (26). The BC360 BRCAness score was calculated as a linear combination of log-transformed expression values from 53 genes (Supplementary Table S3). This score was trained to recapitulate the score of Severson and colleagues (30) in a manner that is robust across gene expression platforms. Normalized gene expression data and signatures discussed in this study have been deposited at European Genome-phenome Archive (https://www.ebi.ac.uk/ega/; EGAS00001004594).

Whole-exome sequencing

A subset of tumors from the IKP211 cohort ($N = 36$) were analyzed for an association between mutation patterns and TIS score. This analysis included 18 patients with disease recurrence and 18 controls without recurrence matched by prognostic variables (age, tumor size, grade, nodal status, treatment). We performed whole-exome sequencing (WES) of tumor and corresponding blood-derived normal DNA (Agilent SureSelect, Illumina) for the identification of single nucleotide variants and small indels. Sequencing reads were aligned to the hg38 reference genome using Burrows-Wheeler Alignment tool (31). Somatic variants were assigned by local assembly and read-to-haplotype alignment in both tumor and normal samples followed by somatic-specific genotyping and filtering performed by GATK mutect2 (32). HLA class-1 genotype calling was performed on the basis of germline WES reads using OptiType (33). Assessment of tumor mutational burden was done based on nonsynonymous coding mutation counting.

Bioinformatic and statistical analyses

Discovery cohort

Gene signature scores and breast cancer key genes *ESR1, PGR, AR*, and *FOXA1* were tested for linear associations with clinical outcome using univariate Cox proportional hazard regression (R library survival). Clinical endpoints were defined as time from diagnosis to the first occurrence of a breast cancer recurrence (local or distant) or secondary breast cancer as breast cancer–free interval (BCFI), or time from diagnosis to the first occurrence of a breast cancer recurrence (local or distant), secondary breast cancer, or death of any cause as event-free survival (EFS). Significance levels in association analyses were corrected for multiple testing using the Benjamini–Hochberg procedure. Conditional inference tree analyses (R library partykit) were performed on significant candidates to determine cutoffs for cohort partitioning with regards to clinical outcome. Clinical associations were tested using log-rank tests in subgroups of lower- and higher-risk patients, and by multivariate Cox models adjusted for additional prognostic factors.

The TIS score distribution was analyzed by Gaussian finite mixture modeling (R library mclust) for the presence of different clusters.

Signatures were clustered using resampling-based consensus clustering (library ConsensusClusterPlus). For this purpose, score distributions were mean-centered and standardized followed by 500 clustering runs (k-means algorithm, euclidean distances). In each run, cluster numbers 2–10 were evaluated on a random subset comprising 80% of the patients. The final clustering of the signatures was based on the consensus matrix. Ward's method was used in all hierarchical clustering analyses. Spearman correlation coefficients and corresponding significance tests for association were calculated to analyze co-expression of signatures.

Independent verification cohort

Normalized and batch-corrected Human Genome U133a/U133 Plus array data were obtained from BreastMark, and BRCAness and TIS scores were calculated. Androgen receptor (AR) expression values were determined by taking the mean of AR-assigned probesets (Bioconductor hgu133a2.db library). Distributions of signature scores and expression values were adjusted to the discovery cohort data by median-centering (Supplementary Fig. S2). In Gaussian finite mixture modeling, possible subdistributions were forced to have equal variance. Clinical associations were tested as in the discovery cohort.

Results

Patient characteristics and PAM50 risk assignments

The IKP211 early breast cancer discovery cohort comprised 612 postmenopausal patients with a median follow-up of 5.5 years and a median age at diagnosis of 65 years (**Table 1**). Univariate Cox regression revealed age, tumor size, nodal status, morphologic grade, ROR, and PAM50 intrinsic subtype as prognostic factors of clinical outcome (**Table 1**), which were adjusted for in multivariate analyses. Using PAM50 subtype-dependent outcomes, we classified patients into higher- and lower-risk groups for recurrence or death (EFS; Supplementary Fig. S3). As expected, LumA patients had most favorable outcomes and were classified as lower-risk. Significantly worse outcomes were observed for the other subtypes which were combined into a higher-risk group: LumB, HR = 1.57 [95% confidence interval (CI), 1.02–2.42]; HER2, HR = 2.44 (95% CI, 1.29–4.60);

Table 1. Patient and tumor characteristics, stratified by PAM50 risk groups and for all patients of the IKP211 discovery cohort.

Characteristics	Lower-risk ($n = 358$)[a]		Higher-risk ($n = 254$)[a]		P[b]	Overall ($n = 612$)		P[c]
	No. of patients	%	No. of patients	%		No. of patients	%	
Follow-up, years					0.69			
Median (range)	5.5 (0.03–12.6)		5.5 (0.03–12.5)			5.5 (0.03–12.6)		
Age, years					0.25			0.001
Median (range)	64.7 (46.2–84.6)		65.4 (48.2–85.3)			65.0 (46.2–85.3)		
ROR					<0.001			0.0001
Median (range)	33 (1–62)		66 (37–97)			47 (1–97)		
Tumor size (cm)					<0.001			0.001
≤2	255	71.2	132	52.0		387	63.2	
2–5	86	24.0	105	41.3		191	31.2	
>5	16	4.5	16	6.3		32	5.2	
Unknown	1	0.3	1	0.4		2	0.3	
Nodal status					0.007			0.04
Negative	279	77.9	169	66.5		463	73.3	
Positive	76	21.2	82	32.3		162	25.6	
Unknown	3	0.8	3	1.2		7	1.1	
Grading					<0.001			0.02
G1	107	29.9	14	5.5		121	19.8	
G2	236	65.9	176	69.3		412	67.3	
G3	12	3.4	62	24.4		74	12.1	
Unknown	3	0.8	2	0.8		5	0.8	
Hormone receptor status								
ER-positive	358	100	249	98.0	0.01	607	99.2	0.88
ER-negative	0	0	5	2.0		5	0.8	
PR-positive	317	88.5	210	82.7	0.04	527	86.1	0.09
PR-negative	41	11.5	44	17.3		85	13.9	
HER2 status					0.02			0.37
Positive	19	5.3	32	12.6		51	8.3	
Negative	338	94.4	221	87.0		559	91.4	
Unknown	1	0.3	1	0.4		2	0.3	
PAM50 subtype					<0.001			0.001
Luminal A	358	100	0	0		358	58.5	
Luminal B	0	0	200	78.7		200	32.7	
HER2	0	0	37	14.6		37	6.0	
Basal	0	0	17	6.7		17	2.8	
Chemotherapy					<0.001			0.09
Adj/neoadjuvant	83	23.2	109	42.9		192	31.4	
None	271	75.7	144	56.7		415	67.8	
Unknown	4	1.1	1	0.4		5	0.8	
Endocrine therapy								0.32
Tamoxifen	111	31.0	52	20.5	0.001	163	26.6	
AI	137	38.3	134	52.8		271	44.3	
Switch	110	30.7	68	26.8		178	29.1	

Abbreviation: Adj, adjuvant.
[a]Risk group classified by PAM50 subtype into lower-risk (LumA) and higher-risk (LumB, Her2, and Basal).
[b]χ^2 or Fisher exact test for differences between the two risk groups.
[c]Cox regression test for an association with clinical outcome.

Basal, HR = 3.48 (95% CI, 1.38–8.78; HRs relative to LumA; Supplementary Fig. S3). Because patient and tumor characteristics differed between the two risk groups except for age (**Table 1**), subsequent outcome analyses were carried out separately according to PAM50-defined risk grouping.

Outcome analyses according to BC360 signatures

Gene signature and selected single-gene expression scores were analyzed for linear associations with BCFI and EFS by univariate Cox modeling (**Fig. 1**; EFS was chosen for data presentation because this endpoint has been more consistently documented in the verification cohort). In the lower-risk/LumA group, there was no significant association following correction for multiple testing except for intrinsic subtype signatures. In contrast, several signatures were observed in the higher-risk group of which those not retaining significance upon correction for multiple testing (mast cell, proliferation, and ROR) were discarded from subsequent analyses: a favorable prognosis was associated with higher expression of pioneer transcription factor and modulator of nuclear hormone receptors (*FOXA1*) and of the androgen receptor (*AR*); poor prognosis was associated with higher score of the "p53 signature" representative for a mutated p53 signaling pathway and of the "BRCAness signature" indicating defects in the DNA damage repair genes *BRCA1* and/or *BRCA2* or associated effector genes (Supplementary Table S3). Stepwise addition of all four "robust" signature scores into a combined Cox model retained *AR* and BRCAness as independent molecular variables. Accordingly, *AR* expression

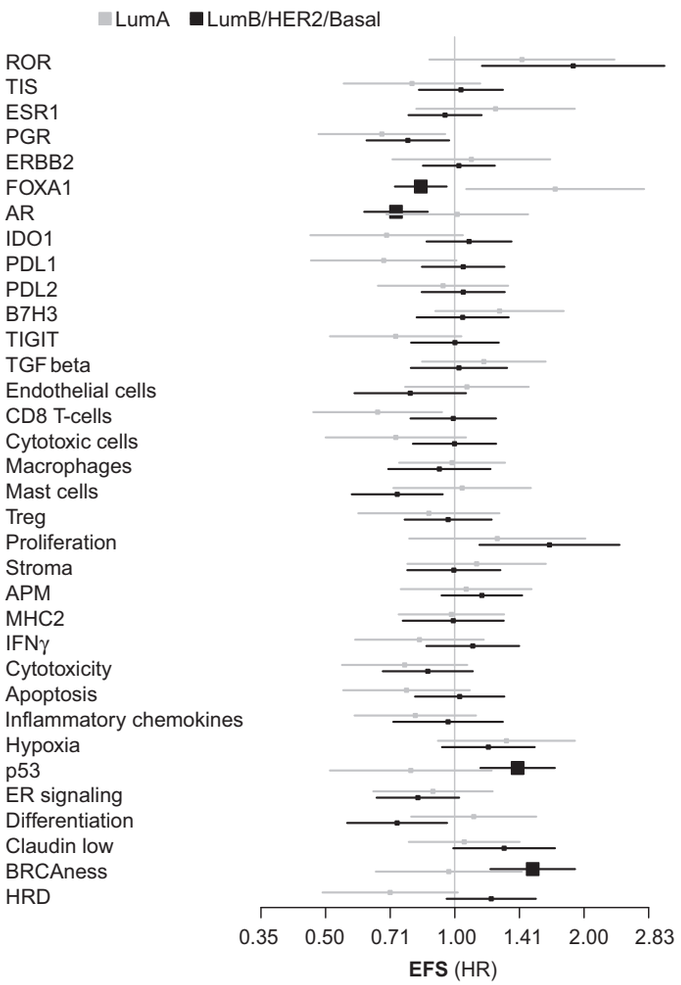

Figure 1.

Univariate Cox regression analysis for linear associations between BC360 signatures/breast cancer key genes and event-free survival (EFS). Expression levels and signature scores were mean-centered and standardized. Separate analyses were done for lower-risk/LumA patients (gray) and for subtypes conferring higher-risk (LumB/HER2/Basal; black). Points show HRs per unit increase in each signature; lines show associated 95% confidence intervals. Values higher/lower than the reference value 1 (= no change; vertical line) indicate an association with increased or reduced risk of recurrence or death, respectively. Large squares indicate significant associations following Benjamini–Hochberg correction for multiple testing. Signature abbreviations: ROR, risk of recurrence; *ESR1*, estrogen receptor 1, *PGR*, progesterone receptor; *ERBB2*, Erb-B2 receptor tyrosine kinase 2; *FOXA1*, Forkhead Box A1; *AR*, androgen receptor; *IDO1*, Indoleamine 2,3-Dioxygenase 1; PDL1/PDL2, programmed cell death 1 ligand 1/2; B7H3, B7 Homolog 3 (*CD276*); TIGIT, T-cell immunoreceptor with Ig and ITIM domains; CD8 T cells, $CD8^+$ T-cell abundance; Treg, regulatory T-cell abundance as measured by *FOXP3* expression; APM, Antigen processing machinery; MHC2, MHC class II antigen presentation; IFNγ, Interferon gamma signaling; *p53*, p53 signature (mutant-like); ER signaling, estrogen receptor signaling; Claudin low, Claudin-low subtype signature; BRCAness, BRCAness signature capturing defects in DNA damage repair genes *BRCA1* and *BRCA2*; HRD, homologous recombination repair status signature.

was associated with a protective prognostic effect (HR = 0.78; 95% CI, 0.65–0.93; $P = 0.006$), whereas an increased BRCAness score was associated with increased risk of recurrence or death (HR = 1.57; 95% CI, 1.08–2.28; $P = 0.017$).

The BRCAness signature is a valid predictor of poor response in higher-risk patients

Because *AR* and BRCAness signatures were associated with EFS, the presence of patient subgroups with different clinical outcome was examined in the discovery cohort and subsequently evaluated in an independent cohort. Conditional inference tree (ctree) analyses using EFS as a response variable identified a cutoff that separated the outcomes of patients with low *AR* expression from those with high expression ($P = 0.0003$). Within the PAM50 higher-risk patient group ($N = 254$), this cutoff separated 27 patients (10.6%) with low *AR* expression and worse outcome from the remaining 227 patients (89.4%) with high expression and better outcome (Log-rank $P =$ 2.1e-06; **Fig. 2A**). Notably, most patients with low *AR* (82%) had undergone endocrine treatment with AI. In contrast, in lower-risk/LumA patients ($N = 358$), no significant difference was observed (**Fig. 2B**). Using the BRCAness signature as a predictor, ctree analysis also identified two patient groups ($P = 0.0025$). In the PAM50 higher-risk group, patients with high BRCAness scores ($N = 9$, 3.5%) who were predominantly treated with AI had worse outcome compared with those with low scores (log-rank $P = 6.5e-08$; **Fig. 2C**). Among patients with high BRCAness scores, 7 of 9 (78%) patients were of the basal subtype and had 40% higher p53 signature scores compared with the low BRCAness group ($P < 0.01$). When applying the same cutoff in the lower-risk/LumA group, no patients with high BRCAness were observed (**Fig. 2D**). We did not observe an influence of chemotherapy on the prognostic value because AR and BRCAness were significantly associated with outcome in both patients with and without additional chemotherapy (Supplementary Table S4). Moreover, correlation analyses showed that BRCAness was only weakly correlated with the proliferation signature ($r = 0.27$; Supplementary Fig. S4).

Using the cutoff values defined in the discovery cohort, worse outcomes of patients with low *AR* (18%) and high BRCAness (7.4%) were confirmed in the higher-risk group (LumB/HER2/Basal) of the BreastMark verification cohort [log-rank P (AR) = 0.03, P (BRCAness) = 0.02; Supplementary Fig. S5A and S5C)]. Likewise, no significant clinical association was observed in the lower-risk/LumA patients of this cohort (Supplementary Fig. S5B and S5D).

We next analyzed both molecular predictors together with prognostic tumor variables in multivariate Cox models: both *AR* (HR = 3.66; 95% CI, 1.74–7.68; $P = 0.001$) and BRCAness (HR = 3.51; 95% CI, 1.17–10.53; $P = 0.025$) were retained as predictors of outcome in the discovery cohort, independently of ROR, tumor size, and age (**Table 2**). The same analysis in the verification cohort retained

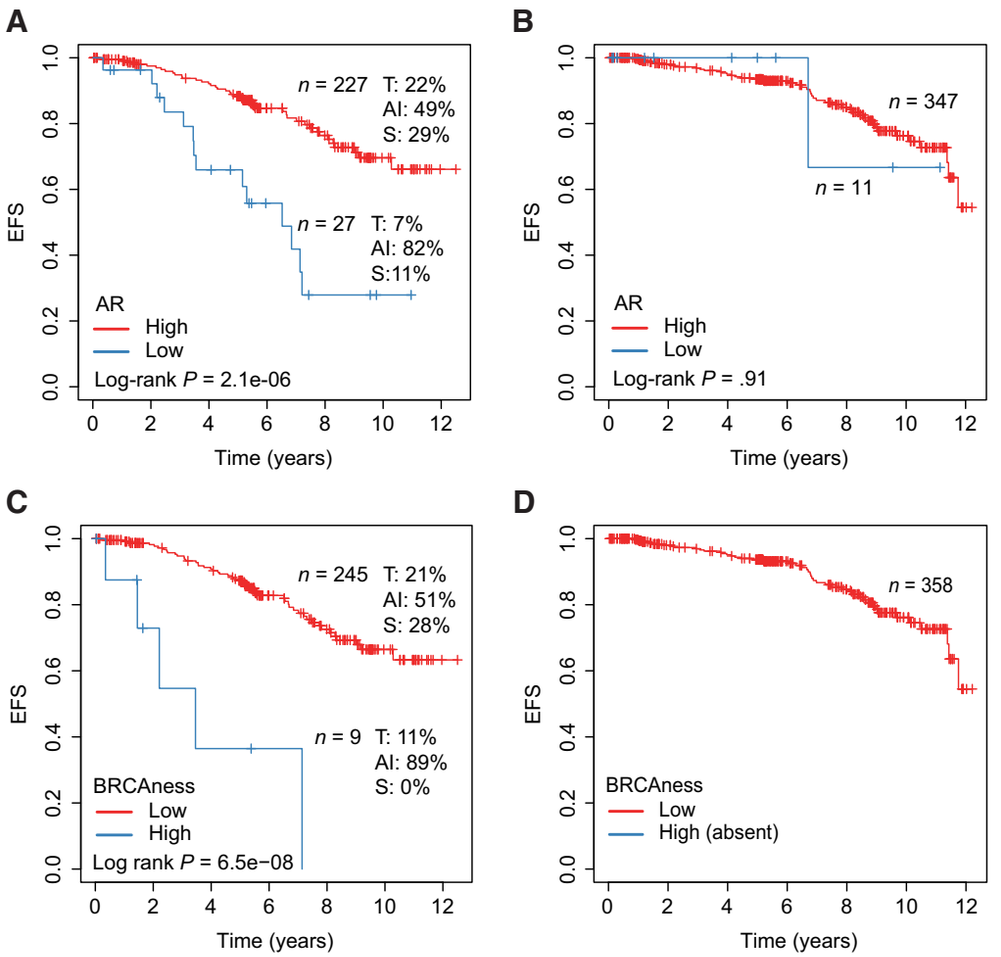

Figure 2.

Associations between androgen receptor (*AR*) and BRCAness gene expression signatures and event-free survival (EFS). Expression scores were dichotomized into high and low expression associated with different risk of recurrence by recursive binary partitioning using conditional inference trees. **A,** Kaplan–Meier curves of higher-risk (LumB/HER2/Basal) patients stratified by *AR*. **B,** Kaplan–Meier curves of lower-risk patients stratified by *AR*. **C,** Kaplan–Meier curves of higher-risk patients stratified by BRCAness. **D,** Kaplan–Meier curves of lower-risk patients stratified by BRCAness. The number of patients per strata and the proportion of patients with antihormonal treatment tamoxifen (T), aromatase inhibitor (AI), and switch (S) are shown in higher-risk patients. Higher-risk patients were defined by LumB, HER2, or Basal, and lower-risk patients were defined by LumA intrinsic subtype.

BRCAness (HR = 2.64; 95% CI, 1.31–5.34; $P = 0.007$) but not *AR* as an independent predictor together with tumor size (**Table 2**). On the basis of this confirmation, BRCAness is considered a molecular predictor of poor outcome in a subgroup of hormone-sensitive early breast cancers that pertains to about 3.5%–7.4% of the higher-risk LumB/HER2/Basal breast cancer subgroup.

Early breast cancers enriched for high TIS scores

Consensus clustering of standardized BC360 signatures across all patients of the discovery cohort revealed four major groups (Supplementary Fig. S6). These were composed of expression signatures of pathways related to tumor immune activity including TIS (cluster A), tumor responsiveness, regulation including BRCAness (cluster B), steroid receptor signaling and differentiation (cluster C), as well as a heterogenous cluster that combines features of cluster A with stroma signals (cluster D). In-depth analysis of the TIS score by Gaussian finite mixture modeling revealed that the TIS score distribution is composed of two different patient groups (Supplementary Fig. S6). In the discovery cohort, 82 patients (13.4%) with increased TIS scores (TIS > 7.52) were separated from the majority of patients with lower TIS values. In the verification cohort, an almost identical distribution has been found with 99 patients (14.7%) forming the patient subgroup with high TIS values (> 7.32; **Fig. 3A** and **B**).

To further characterize TIS-high patients, we scrutinized their tumor characteristics as well as hormonal treatment regimes, and evaluated tumor mutation patterns obtained from WES of 36 patients (**Table 3**). In both the discovery and the independent verification cohort, TIS-high patients were strongly enriched for higher-grade tumors (G2, G3) and higher-risk PAM50 subtypes ($P \leq 0.0001$), whereas tumor size, nodal status, age, and hormonal treatment showed minor or no influences. WES of 28 and 8 patients with low and high TIS scores, respectively, did not reveal any differences between TIS-low and -high with regard to the tumor mutational burden (TMB) of functional mutations. Likewise, there was no difference in the level of

Table 2. Combined Cox proportional hazards model estimates of expression scores and prognostic variables in higher-risk patients of IKP211 (discovery) and BreastMark (verification).

Cohort	Predictor	HR[a] (95% CI)	*P*
IKP211	Age	1.04 (1.00–1.08)	0.019
	ROR	1.03 (1.00–1.05)	0.048
	Tumor size	1.29 (1.04–1.60)	0.02
	BRCAness	3.51 (1.17–10.53)	0.025
	AR	3.66 (1.74–7.68)	0.001
Breastmark	Tumor size	1.69 (1.09–2.64)	0.02
	BRCAness	2.64 (1.31–5.34)	0.007

Note: BRCAness: impaired BRCA function – low (reference) versus high; AR, androgen receptor expression - high (reference) versus low.
[a]HRs adjusted for variables age (continuous); tumor size: ≤2 cm (reference), 2–5 cm, >5 cm; nodal status: categorical, negative and positive; histologic grade: low (reference), medium, high; ROR, risk of recurrence continuous score (0–100) inferred from PAM50 subtype and tumor size (29).

heterozygosity of HLA class 1 genes, or the somatic mutation load of any HLA class 1 or 2 genes (**Table 3**).

Tumors with high TIS scores show increased numbers of TILs

Next, we investigated the relation between TIS and other BC360 immune-response signatures and the level of TILs. There was a strong correlation between TIS and stromal TIL infiltrates ($r = 0.62$; $P < 1e\text{-}30$; **Fig. 3C**). In particular, the 22 patients (3.6%) who had lymphocyte-predominant tumors with ≥50%–60% TILs (**Fig. 3D**) also had the highest TIS scores which ranged consistently (exception of one tumor) above our described cutoff of 7.52. In contrast, the majority of tumors with lower TIL numbers (Supplementary Fig. S7) had TIS ranges clearly below cutoff (**Fig. 3C**). We also observed other TIS-high patients ($N = 59$, 9.7%) with TIL counts in the range of 5%–45% indicating that molecular (TIS) and histologic (TIL) traits only partially overlap. Of the BC360 signatures that inform on immune cell population abundances, that is, cytotoxic cells, $CD8^+$ T cells, macrophages, mast cells, and regulatory T cells (Treg), the strongest positive associations with TILs have been observed with signatures of cytotoxic cells and $CD8^+$ T cells ($r = 0.6$ and $r = 0.58$, respectively, $P < 1e\text{-}30$; Supplementary Fig. S8). Accordingly, TIL count and TIS are strong-positively correlated with the presence of functional antitumor immune effector cells.

Discussion

We present novel gene expression signatures related to BRCAness and immune response that in addition to intrinsic subtype classification may be useful predictors towards improved treatment strategies in patients with hormone receptor–positive early-stage breast cancer. In a cohort of postmenopausal patients, who based on their positive ER and/or PR tumor status had received 5 years of endocrine treatment intrinsic tumor subtypes, were retrospectively assessed with the PAM50 classifier contained in the nCounter BC360 gene expression panel (NanoString). We confirmed the biological heterogeneity of hormone receptor–positive breast cancers with 58% of the tumors showing LumA, 33% LumB, 6% HER2, and 3% Basal subtype characteristics. Using EFS as an outcome measure, we also confirmed the more favorable prognosis of LumA, and the increased recurrence and death risk of LumB/HER2/Basal subtypes (34). Accordingly, intrinsic subtype classification appeared useful to identify patients with an increased risk of recurrence or death potentially in need for adapted treatment strategies.

Following our primary study objective, the BC360 gene expression signatures did not further stratify LumA tumors into different prognostic subgroups; however, an extended breast cancer subcategorization was possible in the higher-risk LumB/HER2/Basal group. Two distinct gene signatures, that is, BRCAness and TIS, were associated with either clinical outcome or the presence of tumor immunogenicity, respectively. The BRCAness signature has been independently confirmed as a strong classifier in that 50% to 55% of patients with a "high" signature score had experienced breast cancer recurrences or death, of which the majority of events occurred within the first 4 years after breast cancer diagnosis. In contrast, only 10% to 20% of LumB/HER2/Basal patients with a low BRCAness score experienced an event during the same time interval. The high BRCAness-associated early event rate identified this patient group of being at a very high risk for recurrence and death, which exceeds that known for aggressive TNBCs (35). Importantly, the BRCAness signature was shown to be prognostic independent of tumor proliferation and chemotherapy, thereby underscoring the clinical relevance of this new stratification. Biologically, BRCAness refers to sporadic tumors that phenocopy the effects of germline *BRCA1* and *BRCA2* mutations known to impair the homologous recombination repair (HRR) of DNA double-strand breaks (36). Commonly, TNBC and the Basal intrinsic breast cancer subtype as well as many other tumor entities are linked with BRCA1 or BRCA2 mutations, or gene alterations (e.g., of *ATM, CHEK2, PALB2, RAD51, FANCA, FANC-F, BLM)* contributing to the BRCAness signature. Moreover, p53 mutations are common features of *BRCA*-mutant sporadic tumors further enhancing the level of genomic instability (37). In line with this, the majority of our patients (78%) with a high BRCAness score have been assigned to the Basal intrinsic subtype and had a 40% increased p53 signature expression (surrogate of mutated p53). While the latter is in line with nonfavorable outcomes reported from p53-mutated tumors treated with AI (38), multivariate analysis in our study did not confirm an independent contribution of impaired p53 to clinical outcome, although this has been suggested in the univariate analysis (**Fig. 1**). The BRCAness/p53 interdependency related to impaired global DNA damage repair (39) may therefore be governed by the BRCAness phenotype. We conclude that the BRCAness signature may be a promising marker to identify those hormone receptor–positive higher-risk patients (predominantly with Basal subtype and p53-mutated tumors) who may benefit from an upgrade of standard chemo-endocrine treatment regimens with therapeutic approaches that interfere with HRR such as platinum salts and PARP inhibitors for the targeting of alternative DNA repair pathways. Further validation of this clinical entity, for example, by reanalyses of large breast cancer trials such as ATAC and BIG1–98, and by BRCAness-stratified response analyses in platinum chemotherapy trials would help to substantiate the clinical relevance.

High TIS scores indicative of an inflamed tumor phenotype have been identified in another 13% to 15% of patients both in the discovery and verification cohort, thereby suggesting an adaptive immune response in these tumors. The finding of putative "hot" tumors in an endocrine treatment cohort is surprising, because until recently ER-positive breast cancers have been perceived as a tumor entity lacking immune responsiveness given a different (adverse) role of TILs in this subgroup compared with their favorable effect on outcome in TNBC and HER2-positive breast cancers (22, 23, 40, 41). Commonly, immune responsiveness has been assessed by IHC-based TIL evaluations (e.g., $CD8^{+/-}$, $FOXP3^{+/-}$). In contrast, the TIS signature used in

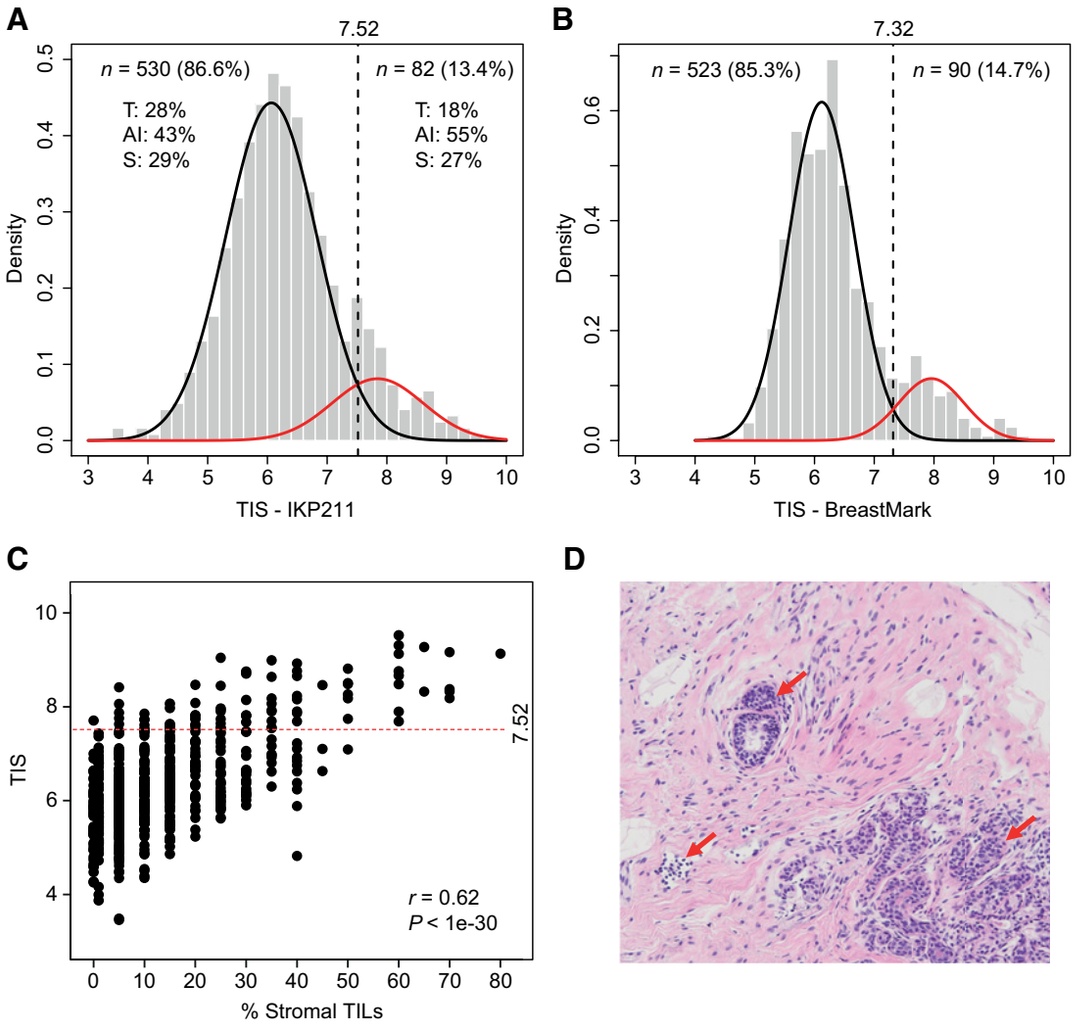

Figure 3.

Distribution of TIS values and its association with tumor-infiltrating immune cells (TIL). **A,** Histogram of the TIS score in the IKP211 discovery cohort. Subpopulations in the distribution of TIS scores (black and red) and the corresponding cutoff (dashed line) were identified by model-based clustering. A subgroup of patients ($n = 82$, 13.4%) was separated with high TIS scores >7.52. Antihormonal treatment modes tamoxifen (T), aromatase inhibitor (AI), and switch (S) in both TIS groups are shown. **B,** In the independent BreastMark verification cohort, TIS scores were centered to the median of the IKP211 cohort. Model-based clustering separated patients ($n = 90$, 14.7%) with high TIS scores >7.32. **C,** TIS scores versus TIL numbers. TIL numbers, rounded to multiples of 5%, indicate the percentage of stroma tissue occupied by TILs. Pearson correlation coefficient and associated *P* value are given. The horizontal dashed line marks the cutoff derived from model-based clustering that separates patients into TIS high/low. **D,** Example of a hematoxylin and eosin–stained section from a tumor of TIL score >60% showing a high number of infiltrating immune cells indicated by red arrows.

our study contains 18 functional genes that predict response to PD-1 pathway blockade by measuring distinct areas of immune biology including IFNγ biology, T-cell exhaustion, T-cell/NK signature, and antigen-presenting cell signature (26). The signature detects a peripherally suppressed, preexisting pan-cancer adaptive immune response that was trained in 11 different tumors (not containing breast cancer) and confirmed in an additional 8 tumor types including TNBC (42). Our finding is in line with two recent studies reporting on the role of immunogenicity in ER-positive breast cancer. Wagner and colleagues identified an immunosuppressed T-cell landscape in high-grade ER-positive tumors by virtue of single-cell proteome analysis (21), which is concordant with our finding of an "inflamed" tumor type in frequently high-grade tumors (i.e., G2 and G3). A clinical study by Anurag and colleagues showed that neoadjuvant AI treatment–resistant LumB breast cancers frequently showed an upregulation of the immune checkpoint components IDO1 and LAG3 (43). Although we did not observe an association of TIS with clinical outcome, it should be noted that the 18-gene TIS classifier includes LAG3 as a marker of T-cell exhaustion and IDO1 as a marker of IFNγ biology (43). Despite the different study context (treatment-naïve vs. treatment-resistant tumors) both findings support the notion that some immunogenic ER-positive tumors adopt evasion mechanisms and are possibly targetable by immune checkpoint inhibition. This treatment option may pertain to hormone receptor–positive tumors that can be easily identified on the basis of their TIS score in primary formalin-fixed, paraffin-embedded tumor specimen.

Table 3. Patient, tumor, and molecular characteristics according to classification into TIS low and high score in the breast cancer cohorts IKP211 (discovery) and BreastMark (verification).

		IKP211 (TIS cutoff >7.52)					BreastMark (TIS cutoff >7.32)				
Characteristic		**TIS low**	**%**	**TIS high**	**%**	***P*[a]**	**TIS low**	**%**	**TIS high**	**%**	***P*[a]**
PAM50	LumA	331	63	27	33	<0.0001	255	49	16	18	<0.0001
	LumB	166	31	34	42		243	47	56	62	
	HER2	27	5	10	12		16	3	12	13	
	Basal	6	1	11	13		9	2	6	7	
Grade	G1	114	22	7	9	<0.0001	128	25	4	4	<0.0001
	G2	359	68	53	65		255	49	37	41	
	G3	53	10	21	26		90	17	40	44	
Nodal status	Negative	398	75	52	63	ns	265	57	39	47	ns
	Positive	130	25	28	34		203	43	44	53	
Tumor size	≤2 cm	337	64	50	61	ns	204	51	26	38	ns
	2–5 cm	163	31	28	34		186	47	40	58	
	>5 cm	29	5	3	4		8	2	3	4	
Age	Median	65.2		62.2		0.03	65		62		ns
HT[b]	TAM	148	28	15	18	ns					
	AI	226	43	45	55						
	Switch	156	29	22	27						
	Whole-exome sequencing ($N = 36$)[c]										
TMB[d]	Mean	116		105		ns					
	Range	19–421		25–325							
HLA class-1[e]	hom	6	21	1	13	ns					
	het	22	79	7	87						
HLA mut[f]	wt	21	75	6	75	ns					
	mut	7	25	2	25						

Abbreviation: ns, not significant.
[a]Two-sided χ^2 test of differences between TIS low and high subgroups.
[b]Hormonal treatment; data not available from BreastMark.
[c]Whole-exome next-generation sequencing analysis of 28 TIS-low and 8 TIS-high tumors of the IKP211 cohort.
[d]Tumor mutational burden given as absolute number of non-synonymous mutations.
[e]HLA-A, B, C gene zygosity deduced from germline whole-exome sequencing: hom, any HLA gene homozygous; het, all HLA genes heterozygous.
[f]Tumor mutations of HLA class -1 and -2 genes: wt, no mutation; mut, somatic mutation present in any HLA gene.

Supportive evidence for a clinical relevance of the TIS score comes from our additional finding of a strong enrichment of TILs in tumors with high TIS levels, which paralleled an increased expression of signatures for CD8+ and cytotoxic cell abundances, corroborating an active status of the adaptive immune system in these tumors. Notably, in a study of pre-therapeutic core biopsies of patients with different breast cancer subtypes, the presence of TILs in TNBC and HER2-positive breast cancers was prognostic for favorable response to neoadjuvant chemotherapy and clinical outcome, whereas TILs in Luminal/HER2-negative tumors were associated with reduced patient survival (23). As in our study the TIS score had no prognostic value in Luminal/HER2-negative patients (data not shown), and TIS and TIL features showed only partial overlap (not all patients with high TIS score had high TIL numbers), the TIS predictor likely selects a patient group that differs from a TIL-based stratification. This is in line with the inherent characteristic of TIS being a predictive marker of response to immune checkpoint blockade rather than a prognostic signature.

To better understand possible mechanisms of the observed immune cell attraction, we assessed the mutational landscape in a small set of tumors. Neither the number of tumor mutations nor loss of heterozygosity and function of HLA class 1 genes were associated with TIS, implicating mechanisms other than mutation-induced neoantigen presentation or loss of HLA heterozygosity (44) for a modulation of immune response in these tumors. Recent studies suggest that mutation-driven pertubations of the MAPK and PI3K pathways are modulators of an immune response in Luminal breast cancer (45, 46). It will be important, therefore, to investigate a putative relationship between the TIS signature and these mutational patterns, which was not possible in the present study due to the limited number of available exomes.

Altogether, the presence of a robust and measurable tumor immune infiltrate along with high TIS levels supports the notion of an inhibited antitumor immune response that may open the route for the combination of standard adjuvant treatment with immune checkpoint blockade in eligible, higher-risk patients. Of note, immune checkpoint blockade by pembrolizumab and atezolizumab has been recently shown to increase progression-free and overall survival in patients with advanced TNBC (24, 25).

We further observed a protective effect between increased *AR* gene expression and longer EFS in higher-risk patients; however, this finding has not been independently confirmed. Currently, this observation remains elusive given the ambivalent reports regarding AR's growth-inhibitory versus -promoting role in breast cancer (47) including a recent negative finding for its prognostic role (48). The controversies are possibly due to the lack of standardization for AR positivity, or failure to distinguish genomic from nongenomic AR signaling, with the latter resulting in PI3K/Akt pathway activation known to contribute to endocrine resistance (49). In our study, FOXA1, the pioneer transcription factor and modulator of nuclear hormone receptors, was the most strongly AR coregulated gene (data not shown), in line with previous observations (50). Therefore, to further explore the role of AR in ER-positive early breast cancer, coexpression of FOXA1 as a driver

of genomic signaling as well as investigations of breast tumors with low AR expression should be considered.

Our data have limitations; the short median follow-up of 5.5 years in the discovery cohort may have impacted the predictive value of gene signatures for late recurrences. However, 43% of the breast cancer recurrences occurred beyond 5 years, which is comparable with the expected 46% in the 10-year observation period of long-term surveys in ER-positive breast cancer (3). Furthermore, differences in gene expression methods between discovery and verification cohorts may have confounded study conclusions. Yet, based on robust normalization in both platforms and comparable distribution of three genes/signatures following median centering (Supplementary Fig. S2), this risk may be minor. The low- versus high-risk of relapse categorization is commonly based on ROR-PAM50; because the absolute numbers of positive lymph nodes were not available, we used subtype-only PAM50 risk categorizations instead. Finally, the observational study design precludes strong conclusions on the clinical utility in homogeneously treated patients. To this end, we confirmed BRCAness as being prognostic independent of chemotherapy thereby substantiating its potential clinical relevance.

In summary, we showed that the next-generation classification of hormone-dependent early breast cancers can be based on novel classifiers for a comprehensive exploration of the tumor's biological characteristics and therapeutic requirements. While initial intrinsic subtype classification allows to select patients at higher risk for recurrence, subsequent testing of BRCAness and TIS gene expression signatures may provide a means to guide adjuvant treatment decisions for the extension of standard chemo-endocrine therapies by agents targeting DNA repair deficiency or immune checkpoints. Such extended versus standard regimen concepts need to be further substantiated by suitable clinical investigations, for example, the assessment of clinical responses in biomarker-selected neoadjuvantly treated patients.

Appendix

Patient recruitment was carried out by the German Tamoxifen and AI Clinicians Group

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Disclosure of Potential Conflicts of Interest

W. Schroth reports grants from Robert Bosch Stiftung, Deutsche Forschungsgemeinschaft DFG, and Interfaculty Center for Pharmacogenomics and Drug Research ICEPHA during the conduct of the study. F.A. Büttner reports grants from Robert Bosch Stiftung during the conduct of the study. S. Kandabarau reports grants from Robert Bosch Stiftung during the conduct of the study. R. Hoppe reports grants from Robert Bosch Stiftung and Interfaculty Center for Pharmacogenomics and Drug Research ICEPHA during the conduct of the study. J. Kumbrink reports personal fees and other from Novartis (advisory board, reimbursement for travel and accommodation) and Quality Initiative in Pathology (QuIP; reimbursement for travel), as well as personal fees from AstraZeneca (reimbursement for travel and accommodation) and Roche (speaker honoraria) outside the submitted work. T. Kirchner reports other from Amgen, AstraZeneca, Merck, MSD, Novartis, Pfizer, and Roche outside the submitted work. H.A. Brauer reports other from NanoString (employee) outside the submitted work. P.A. Fasching reports grants and personal fees from Novartis and personal fees from Astrazeneca (advisory board) during the conduct of the study, as well as personal fees from Pfizer, Daiichi-Sankyo, Eisai, Merck Sharp & Dohme, Lilly, Pierre Fabre, and Seattle Genetics, and grants from Cepheid outside the submitted work. T.E. Mürdter reports grants from Robert Bosch Stiftung, Deutsche Forschungsgemeinschaft, and Interfaculty Center for Pharmacogenomics and Drug Research ICEPHA, University of Tübingen during the conduct of the study. M. Schwab reports grants from Robert Bosch Stiftung (Stuttgart, Germany), BMBF (grant 01ZP0502), Interfaculty Center for Pharmacogenomics and Drug Research (ICEPHA) University of Tübingen Germany, German Cancer Consortium (DKTK) and German Cancer Research Center Tuebingen Germany, and Deutsche Forschungsgemeinschaft (DFG) im Rahmen der Exzellenzstrategie des Bundes und der Länder - EXC 2180 - 390900677 during the conduct of the study; in addition, M. Schwab has a patent for EP2018/080307, and pending patents for EP19169035.3 and for PCT/EP2020/056398; and wishes to disclose evaluations with HongKong RIF 2018/19/20 and EU H2020-SC1-2019-Two-Stage-RTD, an Editor-in-Chief role at "Pharmacogenetics and Genomics" and "Drug Research", and honoraria for oral presentations at academically organized congresses and meetings and for lectures from ALL Academy Frankfurt, InfectoPharm Bonn, and CED Service GmbH Mannheim. German Tamoxifen and AI Clinicians Group reports grants from Robert Bosch Stiftung and Federal Ministry of Education and Research, Germany during the conduct of the study. H. Brauch reports other from NanoString Technologies during the conduct of the study; this work is based on a colloaboration with NanoString Technologies as evident from the named coauthors Heather Ann Brauer, Yuqi Ren, and David Henderson. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

W. Schroth: Conceptualization, resources, data curation, formal analysis, funding acquisition, methodology, writing-original draft, project administration, writing-review and editing. **F.A. Büttner:** Conceptualization, resources, data curation, software, formal analysis, methodology, writing-review and editing. **S. Kandabarau:** Software, formal analysis, methodology. **R. Hoppe:** Funding acquisition, investigation, writing-review and editing. **P. Fritz:** Data curation, formal analysis, investigation, visualization, methodology, writing-review and editing. **J. Kumbrink:** Resources, methodology, writing-review and editing. **T. Kirchner:** Resources, methodology, writing-review and editing. **H.A. Brauer:** Resources, data curation, software, formal analysis, supervision, methodology, writing-review and editing. **Y. Ren:** Resources, data curation, software, formal analysis, methodology. **D. Henderson:** Resources, data curation, software, formal analysis, supervision, methodology. **S.F. Madden:** Resources, data curation, software, formal analysis, validation, methodology, writing-review and editing. **G. Sauer:** Resources, writing-review and editing. **T. Fehm:** Resources, writing-review and editing. **D. Wallwiener:** Resources. **P.A. Fasching:** Resources, data curation, writing-review and editing. **T. Mürdter:** Data curation, supervision, funding acquisition, project administration, writing-review and editing. **M. Schwab:** Resources, funding acquisition, writing-original draft, project administration,

writing-review and editing. **German Tamoxifen and AI Clinicians Group:** Resources, data curation. **H. Brauch:** Conceptualization, resources, funding acquisition, writing-original draft, project administration, writing-review and editing.

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References

1. Burstein HJ, Lacchetti C, Anderson H, Buchholz TA, Davidson NE, Gelmon KA, et al. Adjuvant endocrine therapy for women with hormone receptor-positive breast cancer: ASCO clinical practice guideline focused update. J Clin Oncol 2019;37:423–38.
2. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Aromatase inhibitors versus tamoxifen in early breast cancer: patient-level meta-analysis of the randomised trials. Lancet 2015;386:1341–52.
3. Pan H, Gray R, Braybrooke J, Davies C, Taylor C, McGale P, et al. 20-year risks of breast-cancer recurrence after stopping endocrine therapy at 5 years. N Engl J Med 2017;377:1836–46.
4. Schroth W, Goetz MP, Hamann U, Fasching PA, Schmidt M, Winter S, et al. Association between CYP2D6 polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen. JAMA 2009;302: 1429–36.
5. Province MA, Goetz MP, Brauch H, Flockhart DA, Hebert JM, Whaley R, et al. CYP2D6 genotype and adjuvant tamoxifen: meta-analysis of heterogeneous study populations. Clin Pharmacol Ther 2014;95:216–27.
6. Ingle JN, Buzdar AU, Schaid DJ, Goetz MP, Batzler A, Robson ME, et al. Variation in anastrozole metabolism and pharmacodynamics in women with early breast cancer. Cancer Res 2010;70:3278–86.
7. Ingle JN, Cairns J, Suman VJ, Shepherd LE, Fasching PA, Hoskin TL, et al. Anastrozole has an association between degree of estrogen suppression and outcomes in early breast cancer and is a ligand for estrogen receptor α. Clin Cancer Res 2020;26:2986–96.
8. Ma CX, Reinert T, Chmielewska I, Ellis MJ. Mechanisms of aromatase inhibitor resistance. Nat Rev Cancer 2015;15:261–75.
9. Jeselsohn R, Buchwalter G, De Angelis C, Brown M, Schiff R. ESR1 mutations-a mechanism for acquired endocrine resistance in breast cancer. Nat Rev Clin Oncol 2015;12:573–83.
10. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and anti-hormonal resistance in breast cancer. J Clin Oncol 2007;25:5815–24.
11. Ingle JN, Xie F, Ellis MJ, Goss PE, Shepherd LE, Chapman J-AW, et al. Genetic polymorphisms in the long noncoding RNA MIR2052HG offer a pharmacogenomic basis for the response of breast cancer patients to aromatase inhibitor therapy. Cancer Res 2016;76:7012–23.
12. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000;406:747–52.
13. Cancer Genome Atlas Network, Koboldt DC, Fulton RS, McLellan MD, Schmidt H, Kalicki-Veizer J, et al. Comprehensive molecular portraits of human breast tumours. Nature 2012;490:61–70.
14. Curtis C, Shah SP, Chin S-F, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 2012;486:346–52.
15. Ellis MJ, Perou CM. The genomic landscape of breast cancer as a therapeutic roadmap. Cancer Discov 2013;3:27–34.
16. Varga Z, Sinn P, Seidman AD. Summary of head-to-head comparisons of patient risk classifications by the 21-gene Recurrence Score® (RS) assay and other genomic assays for early breast cancer. Int J Cancer 2019;145:882–93.
17. Sparano JA, Gray RJ, Ravdin PM, Makower DF, Pritchard KI, Albain KS, et al. Clinical and genomic risk to guide the use of adjuvant therapy for breast cancer. N Engl J Med 2019;380:2395–405.
18. Bartlett JMS, Sgroi DC, Treuner K, Zhang Y, Ahmed I, Piper T, et al. Breast Cancer Index and prediction of benefit from extended endocrine therapy in breast cancer patients treated in the Adjuvant Tamoxifen-To Offer More? (aTTom) trial. Ann Oncol 2019;30:1776–83.
19. Buus R, Sestak I, Kronenwett R, Denkert C, Dubsky P, Krappmann K, et al. Comparison of EndoPredict and EPclin with oncotype DX recurrence score for prediction of risk of distant recurrence after endocrine therapy. J Natl Cancer Inst 2016;108:djw149.
20. Wallden B, Storhoff J, Nielsen T, Dowidar N, Schaper C, Ferree S, et al. Development and verification of the PAM50-based Prosigna breast cancer gene signature assay. BMC Med Genomics 2015;8:54.
21. Wagner J, Rapsomaniki MA, Chevrier S, Anzeneder T, Langwieder C, Dykgers A, et al. A single-cell atlas of the tumor and immune ecosystem of human breast cancer. Cell 2019;177:1330–45.
22. Ali HR, Provenzano E, Dawson S-J, Blows FM, Liu B, Shah M, et al. Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. Ann Oncol 2014;25:1536–43.
23. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol 2018;19:40–50.
24. Nanda R, Chow LQM, Dees EC, Berger R, Gupta S, Geva R, et al. Pembrolizumab in patients with advanced triple-negative breast cancer: phase Ib KEYNOTE-012 study. J Clin Oncol 2016;34:2460–7.
25. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. N Engl J Med 2018;379:2108–21.
26. Ayers M, Lunceford J, Nebozhyn M, Murphy E, Loboda A, Kaufman DR, et al. IFN-γ–related mRNA profile predicts clinical response to PD-1 blockade. J Clin Invest 2017;127:2930–40.
27. Madden SF, Clarke C, Gaule P, Aherne ST, O'Donovan N, Clynes M, et al. BreastMark: an integrated approach to mining publicly available transcriptomic datasets relating to breast cancer outcome. Breast Cancer Res 2013;15:R52.
28. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol 2015;26: 259–71.
29. Parker JS, Mullins M, Cheang MCU, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol 2009;27:1160–7.
30. Severson TM, Wolf DM, Yau C, Peeters J, Wehkam D, Schouten PC, et al. The BRCA1ness signature is associated significantly with response to PARP inhibitor treatment versus control in the I-SPY 2 randomized neoadjuvant setting. Breast Cancer Res 2017;19:99.
31. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009;25:1754–60.
32. Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat Biotechnol 2013;31:213–9.
33. Szolek A, Schubert B, Mohr C, Sturm M, Feldhahn M, Kohlbacher O. OptiType: precision HLA typing from next-generation sequencing data. Bioinformatics 2014;30:3310–6.
34. Ohnstad HO, Borgen E, Falk RS, Lien TG, Aaserud M, Sveli MAT, et al. Prognostic value of PAM50 and risk of recurrence score in patients with early-stage breast cancer with long-term follow-up. Breast Cancer Res 2017;19:120.

35. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res 2007;13:4429–34.
36. Lord CJ, Ashworth A. BRCAness revisited. Nat Rev Cancer 2016;16:110–20.
37. Turner N, Tutt A, Ashworth A. Hallmarks of "BRCAness" in sporadic cancers. Nat Rev Cancer 2004;4:814–9.
38. Ellis MJ, Ding L, Shen D, Luo J, Suman VJ, Wallis JW, et al. Whole-genome analysis informs breast cancer response to aromatase inhibition. Nature 2012; 486:353–60.
39. Hartman A-R, Ford JM. BRCA1 and p53: compensatory roles in DNA repair. J Mol Med 2003;81:700–7.
40. Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, Shulman LN, et al. Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. J Clin Oncol 2014;32:2959–66.
41. Salgado R, Denkert C, Campbell C, Savas P, Nuciforo P, Nucifero P, et al. Tumor-infiltrating lymphocytes and associations with pathological complete response and event-free survival in HER2-positive early-stage breast cancer treated with lapatinib and trastuzumab: a secondary analysis of the NeoALTTO Trial. JAMA Oncol 2015;1:448–54.
42. Wallden B, Pekker I, Popa S, Dowidar N, Sullivan A, Hood T, et al. Development and analytical performance of a molecular diagnostic for anti-PD1 response on the nCounter Dx Analysis System. J Clin Oncol 2016;34:3034–4.
43. Anurag M, Zhu M, Huang C, Vasaikar S, Wang J, Hoog J, et al. Immune checkpoint profiles in luminal B breast cancer (alliance). J Natl Cancer Inst 2020; 112:737–46.
44. McGranahan N, Rosenthal R, Hiley CT, Rowan AJ, Watkins TBK, Wilson GA, et al. Allele-specific HLA loss and immune escape in lung cancer evolution. Cell 2017;171:1259–71.
45. Hendrickx W, Simeone I, Anjum S, Mokrab Y, Bertucci F, Finetti P, et al. Identification of genetic determinants of breast cancer immune phenotypes by integrative genome-scale analysis. OncoImmunology 2017;6:1–19.
46. Sobral-Leite M, Salomon I, Opdam M, Kruger DT, Beelen KJ, van der Noort V, et al. Cancer-immune interactions in ER-positive breast cancers: PI3K pathway alterations and tumor-infiltrating lymphocytes. Breast Cancer Res 2019;21:90.
47. Ricciardelli C, Bianco-Miotto T, Jindal S, Butler LM, Leung S, McNeil CM, et al. The magnitude of androgen receptor positivity in breast cancer is critical for reliable prediction of disease outcome. Clin Cancer Res 2018;24: 2328–41.
48. Kensler KH, Regan MM, Heng YJ, Baker GM, Pyle ME, Schnitt SJ, et al. Prognostic and predictive value of androgen receptor expression in post-menopausal women with estrogen receptor-positive breast cancer: results from the Breast International Group Trial 1–98. Breast Cancer Res 2019; 21:30.
49. Chia K, Milioli H, Portman N, Laven-Law G, Coulson R, Yong A, et al. Non-canonical AR activity facilitates endocrine resistance in breast cancer. Endocr Relat Cancer 2019;26:251–64.
50. Rangel N, Fortunati N, Osella-Abate S, Annaratone L, Isella C, Catalano MG, et al. FOXA1 and AR in invasive breast cancer: new findings on their co-expression and impact on prognosis in ER-positive patients. BMC Cancer 2018; 18:703.