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PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF THE INTRINSIC MOLECULAR SUBTYPES OF BREAST CANCER

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Title: PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF THE INTRINSIC MOLECULAR SUBTYPES OF BREAST CANCER

Introduction:

Implementation of screening/prevention programs and novel treatment strategies is decreasing breast cancer mortality. However, more than 120,000 estimated deaths due to breast cancer are expected annually in the US and Europe combined. A plausible explanation for this scenario is, in part, that we still lack a complete enough picture of the biologic heterogeneity of breast cancers with respect to molecular alterations, treatment sensitivity, and cellular composition. Importantly, this complexity is not entirely reflected by the main clinical parameters (age, node status, tumor size, histological grade) and pathological markers (estrogen receptor [ER], progesterone receptor [PR] and human epidermal growth factor receptor 2 [HER2]), all of which are routinely used in the clinic to stratify patients for prognostic predictions and to select treatments.

Studies based on global gene expression analyses have provided additional insights into this complex scenario. During the last 10 years, four molecular 'intrinsic' subtypes of breast cancer (Luminal A, Luminal B, HER2-enriched, and Basal-like) and a Normal Breast-like group have been identified and intensively studied. Known as the 'intrinsic subtypes of breast cancer', these groups of tumors have revealed critical differences in incidence, survival, and response to treatment. Importantly, the information provided by the intrinsic subtypes complements and expands the information provided by classical clinical-pathological markers.

Current knowledge of the biology of breast cancer has provided the basis of the various successful adjuvant and neoadjuvant treatment strategies: endocrine therapy for hormone receptor (HR)-positive disease (with or without chemotherapy), anti-HER2 therapies such as trastuzumab in combination or sequentially after chemotherapy for HER2+ disease, and chemotherapy for patients with triple-negative disease. However, the biological diversity displayed by the breast cancer intrinsic subtypes indicate that further sub-classification of patients into different treatment groups should be considered.

The HR+/HER2− group of tumors is mainly composed of two subtypes: Luminal A (good prognosis, chemoresistant and endocrine sensitive) and Luminal B (poor prognosis, mainly chemoresistant and endocrine less sensitive). As discussed above, a main difference between A vs. B is proliferation status, which is low in Luminal A and high in Luminal B tumors. In this context, genomic prognostic assays such as the OncoTypeDX and the MammaPrint signature (or even the pathological marker Ki-67) have the ability to identify tumors with high risk of recurrence, which are mainly Luminal B tumors. An important issue here will be to find which ER+ patients benefit from chemotherapy. As suggested by data from neoadjuvant clinical trials, Luminal B tumors benefit more from chemotherapy than Luminal A tumors, although only less than ∼20% of Luminal B patients eventually achieve a pCR. This increased benefit with the administration of chemotherapy in Luminal B is concordant with data coming from NSABP-B20 trial where only node-negative HR+ patients with high OncoTypeDX RS benefited from adjuvant chemotherapy.

In the HR+/HER2+ group of tumors, two subtypes are mainly identified: Luminal B and HER2-enriched. Here a major challenge will be to elucidate differences between the two molecular subtypes in terms of efficacy of chemotherapy, anti-hormonal therapy, and anti-HER2 therapy. For example, are HR+/HER2+/Luminal B tumors less or more sensitive to anti-HER2 therapies than HR+/HER2+/HER2-enriched tumors, and do they respond better to anti-hormonal therapies than HR+/HER2+/HER2-enriched tumors?

Within HR−/HER2+ tumors, ~50–88% of these fall into the HER2-enriched subtype, followed by the other poor prognostic subtypes. Here the challenge will be to determine if HR−/HER2+ that are not of the HER2-enriched subtype, benefit from anti-HER2 therapies, and if HER2+ tumors that are not of the HER2-enriched subtype show similar or different response rates to trastuzumab when compared to HER2+/HER2-enriched tumors. Finally, within triple-negative disease, Basal-like and Claudin-low are the most frequent subtypes identified. Further studies focusing on the efficacy of particular chemotherapies and/or targeted therapies such as the PARP inhibitors and/or anti-CSC therapies in these subgroups of patients are warranted. It will be important to determine if Basal-like and Claudin-low tumors show similar responses to common therapies as they may given their expression similarities, or they may not given their differences including vast differences in proliferation rates.

Summary of Results:

In the first study, we evaluated the ability of six clinically relevant genomic signatures to predict relapse in patients with ER+ tumors treated with adjuvant tamoxifen only. To accomplish this, we combined four microarray datasets, and we evaluated research-based versions of PAM50 intrinsic subtyping and risk of relapse (PAM50-ROR) score, 21-gene recurrence score (OncotypeDX), Mammaprint, Rotterdam 76 gene, index of sensitivity to endocrine therapy (SET) and an estrogen-induced gene set. Distant relapse-free survival (DRFS) was estimated by Kaplan–Meier and log-rank tests, and multivariable analyses were done using Cox regression analysis. Harrell's C-index was also used to estimate performance.

Our results showed that all signatures were prognostic in patients with ER+ node-negative tumors, whereas most were prognostic in ER+ node-positive disease. Among the signatures evaluated, PAM50-ROR, OncotypeDX, Mammaprint and SET were consistently found to be independent predictors of relapse. A combination of all signatures significantly increased the performance prediction. Importantly, low-risk tumors (>90% DRFS at 8.5 years) were identified by the majority of signatures only within node-negative disease, and these tumors were mostly luminal A (78%–100%).

Thus, we concluded that most established genomic signatures were successful in outcome predictions in ER+ breast cancer and provided statistically independent information. From a clinical perspective, multiple signatures combined together most accurately predicted outcome, but a common finding was that each signature identified a subset of luminal A patients with node-negative disease who might be considered suitable candidates for adjuvant endocrine therapy alone.

In our second study, we showed that three genes (i.e. biomarkers) do not fully recapitulate the entire biological diversity displayed by breast cancer, and that the PAM50 subtype predictor is better. The reason behind this study is that it has recently been proposed that a three-gene model (SCMGENE) that measures ESR1, ERBB2, and AURKA identifies the major breast cancer intrinsic subtypes and provides robust discrimination for clinical use in a manner very similar to a 50-gene subtype predictor (PAM50). However, the clinical relevance of both predictors was not fully explored, which is needed given that a ~30 % discordance rate between these two predictors was observed.

Using the same datasets and subtype calls provided by Haibe-Kains and colleagues, we compared the SCMGENE assignments and the research-based PAM50 assignments in terms of their ability to (1) predict patient outcome, (2) predict pathological complete response (pCR) after anthracycline/taxane-based chemotherapy, and (3) capture the main biological diversity displayed by all genes from a microarray. In terms of survival predictions, both assays provided independent prognostic information from each other and beyond the data provided by standard clinical-pathological variables; however, the amount of prognostic information was found to be significantly greater with the PAM50 assay than the SCMGENE assay. In terms of chemotherapy response, the PAM50 assay was the only assay to provide independent predictive information of pCR in multivariate models. Finally, compared to the SCMGENE predictor, the PAM50 assay explained a significantly greater amount of gene expression diversity as captured by the two main principal components of the breast cancer microarray data. Our results show that classification of the major and clinically relevant molecular subtypes of breast cancer are best captured using larger gene panels.

Finally, in our third study, we improved the corrent immunohistochemical (IHC)-based definitions of luminal A breast cancer. The reason behind this study is that Luminal A and B IHC-based definitions are imperfect when compared with multigene expression-based assays. To accomplish this, we collected gene expression and pathologic features from primary tumors across five independent cohorts: British Columbia Cancer Agency (BCCA) tamoxifen-treated only, Grupo Español de Investigación en Cáncer de Mama 9906 trial, BCCA no systemic treatment cohort, PAM50 microarray training data set, and a combined publicly available microarray data set. Optimal cutoffs of percentage of progesterone receptor (PR) -positive tumor cells to predict survival were derived and independently tested. Multivariable Cox models were used to test the prognostic significance.

Our results showed that the clinicopathologic comparisons among luminal A and B subtypes consistently identified higher rates of PR positivity, human epidermal growth factor receptor 2 (HER2) negativity, and histologic grade 1 in luminal A tumors. Quantitative PR gene and protein expression were also found to be significantly higher in luminal A tumors. An empiric cutoff of more than 20% of PR-positive tumor cells was statistically chosen and proved significant for predicting survival differences within IHC-defined luminal A tumors independently of endocrine therapy administration. Finally, no additional prognostic value within hormonal receptor (HR) -positive/HER2-negative disease was observed with the use of the IHC4 score when intrinsic IHC-based subtypes were used that included the more than 20% PR-positive tumor cells and vice versa. We concluded that semiquantitative IHC expression of PR adds prognostic value within the current IHC-based luminal A definition by improving the

identification of good outcome breast cancers. The new proposed IHC-based definition of luminal A tumors is HR positive/HER2 negative/Ki-67 less than 14%, and PR more than 20%.

Overall, these results suggest that the information provided by the intrinsic subtypes, when combined with the current clinical-pathological markers, helps to further explain the biological complexity of breast cancer, increases the efficacy of current and novel therapies, and ultimately improves outcomes for breast cancer patients.

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Concordance among gene expression-based predictors for ER-positive breast cancer treated with adjuvant tamoxifen

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Background: ER-positive (ER+) breast cancer includes all of the intrinsic molecular subtypes, although the luminal A and B subtypes predominate. In this study, we evaluated the ability of six clinically relevant genomic signatures to predict relapse in patients with ER+ tumors treated with adjuvant tamoxifen only.

Methods: Four microarray datasets were combined and research-based versions of PAM50 intrinsic subtyping and risk of relapse (PAM50-ROR) score, 21-gene recurrence score (OncotypeDX), Mammaprint, Rotterdam 76 gene, index of sensitivity to endocrine therapy (SET) and an estrogen-induced gene set were evaluated. Distant relapse-free survival (DRFS) was estimated by Kaplan–Meier and log-rank tests, and multivariable analyses were done using Cox regression analysis. Harrell's C-index was also used to estimate performance.

Results: All signatures were prognostic in patients with ER+ node-negative tumors, whereas most were prognostic in ER+ node-positive disease. Among the signatures evaluated, PAM50-ROR, OncotypeDX, Mammaprint and SET were consistently found to be independent predictors of relapse. A combination of all signatures significantly increased the performance prediction. Importantly, low-risk tumors (>90% DRFS at 8.5 years) were identified by the majority of signatures only within node-negative disease, and these tumors were mostly luminal A (78%–100%).

Conclusions: Most established genomic signatures were successful in outcome predictions in ER+ breast cancer and provided statistically independent information. From a clinical perspective, multiple signatures combined together most accurately predicted outcome, but a common finding was that each signature identified a subset of luminal A patients with node-negative disease who might be considered suitable candidates for adjuvant endocrine therapy alone.

Key words: breast cancer, genomics, luminal, mammaprint, oncotype, PAM50

introduction

Gene expression-based assays have been developed that can successfully predict outcomes in early-stage ER-positive (ER+) breast cancer beyond standard clinicopathological variables [1–5]. OncotypeDX recurrence score (GHI)[2] and Mammaprint® (NKI70)[3] are clinically available and currently being evaluated in two large prospective clinical trials (TAILORx and MINDACT) [6, 7]. Since then, other prognostic predictors such as the Rotterdam 76-gene signature (ROT76) [8, 9] and the risk of relapse (ROR) score based on the PAM50 subtype assay [10] have been developed using two different node-negative and adjuvant treatment-naive populations.

Previous studies have also shown that many of these expression signatures are concordant for predicting outcomes [11, 12]. However, it is currently unknown if these findings are still valid in a more contemporary ER+ population treated with adjuvant endocrine therapy only [13]. Moreover, recent signatures specifically designed to track hormonal responsiveness, such as the estrogen-induced gene set (IE-IIE) [14] and the genomic index of sensitivity to endocrine therapy (SET) [15], can also predict survival in early-stage ER+ disease. Thus, estrogen-regulated gene signatures could be tracking ER + tumors with high endocrine sensitivity.

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From a clinical perspective, genomic assays are helping to identify patients with early-stage ER+ breast cancers who do not need chemotherapy and are effectively treated with adjuvant endocrine agents alone. Alternatively, they could identify groups of patients with ER+ tumors who are more likely to be biologically homogenous and/or who might benefit from specific treatment strategies. In this report, we evaluated the relapse prediction abilities of six independent genomic signatures using a cohort of ER-positive breast cancer patients treated with adjuvant tamoxifen only.

methods

patients and samples

Four different publicly available microarray datasets were combined together to create a single large set of 594 ER+ patients, all of whom received appropriate local therapy and adjuvant tamoxifen only (see supplemental Figure S1, available at *Annals of Oncology* online). Thousand fifty-three Affymetrix U133A CEL files from various publicly available microarray datasets (GSE17705 [MDACC298] [15], GSE6532 [LOI327] [16, 17], GSE12093 [ZHANG136] [18], GSE1456 [PAWITAN159] [19] and MDACC133 [20]) were processed using MAS 5.0 (R/Bioconductor) to generate probe-level intensities with a median array intensity of 600, and each expression value was $\log_2$ transformed. To batch correct the gene expression data [21, 22], the probeset medians in each individual dataset were adjusted to the MDACC133 reference set accounting for differences in the proportion of clinical ER+ / – samples; after batch correction, all ER– tumors were removed, as were all ER+ tumors not treated with tamoxifen-only, thus leaving 594 tumors per microarrays.

genomic predictors

The following gene expression signatures were evaluated using the combined microarray dataset: GHI [2], NKI70 [3], ROT76 [8], IE-IIE [14], SET [15] and PAM50 [10] (supplemental Table S1, available at *Annals of Oncology* online). Each signature was evaluated as a continuous variable and as group categories according to the published cut-offs [2, 3, 8, 10, 14, 15]. Briefly, the intrinsic subtypes, the risk of relapse based on subtype (PAM50-RORS), the ROR based on subtype and proliferation (PAM50-RORP) and the proliferation index (PAM50-PROLIF) were identified using the PAM50 subtype assay [10]. The PAM50-PROLIF index is the mean expression of 11 PAM50 proliferation-related genes of the PAM50 assay [23]. GHI and NKI70 were evaluated as previously described [12]. For the IE-IIE signature, we calculated the Spearman correlation to the two training centroids (IE and IIE) as described by Oh et al. [14]; samples with a correlation ratio to the IE centroid/IIE centroid >1.0 were assigned to the IE group and the rest to the IIE group. Finally, for the ROT76 and SET signatures, all Affymetrix U133A probes were evaluated as described in both publications, respectively [8, 15]. The list of gene and/or probes, the scores and the group categories for each signature can be obtained from supplemental data, available at *Annals of Oncology* online.

To further explore the PAM50, results were obtained from combining the microarray dataset with a qunatitative RT-PCR (qRT-PCR) dataset of 786 ER+ breast cancer patients treated with adjuvant tamoxifen only from Nielsen et al. [23] (Nielsen series).

statistical analysis

Distant relapse-free survival (DRFS) estimates were from the Kaplan–Meier curves and tests of differences by the log-rank test. The DRFS follow-up time was censored at 8.5 years since it was the longest follow-up time in the PAWI159 [19] dataset. Univariate and multivariable analyses (MVA) were calculated using a Cox proportional regression model.

MVA prognostic models including all the signatures as independent continuous variables were built and assessed using a Cox model with the penalized least absolute shrinkage and selection operator (LASSO) method approach [24]. In each case, a training set (2/3 of the dataset) was randomly used to build a model, which was then applied to the testing set (i.e. the remaining 1/3). We repeated this procedure 200 times as previously carried out [5]. In each testing set, the prognostic performance of each model and each individual signature was estimated by calculating the concordance index (C-index) [25]. All statistical computations were carried out in R v.2.8.1 (http://cran.r-project.org).

results

clinicopathological characteristics of the combined microarray and qRT-PCR PAM50 dataset

We created a large dataset of 1380 ER-positive breast cancer patients treated with adjuvant tamoxifen only using publicly available microarray data ($n = 594$) and PAM50 qRT-PCR data only ($n = 786$) from the Nielsen series [4, 15–19]. Six hundred and ten and 699 patients were identified as having node-negative and node-positive disease, respectively (Table 1). As expected, luminal subtypes predominated ($n = 1171$, 84.9%). Non-luminal subtypes (HER2-enriched and basal-like) represented 9.1% ($n = 125$) of the patients. The normal breast-like samples were not further considered as these specimens are predominantly composed of normal breast tissue, which precludes the correct assignment to a tumor subtype for meaningful outcome predictions [1, 10].

The PAM50 intrinsic subtypes were prognostic for DRFS within node-negative and node-positive patients (Figure 1A and B). In node-negative disease, luminal A tumors showed a better outcome than luminal B [hazard ratio (HR) = 0.313, $P < 0.0001$], HER2-enriched (HR = 0.256, $P < 0.001$) and basal-like (HR = 0.168, $P < 0.001$) subtypes. However, no statistical significant differences in DRFS were observed among the poor prognostic luminal B, HER2-enriched and basal-like subtypes. In node-positive disease, the PAM50 subtypes were also prognostic; of note, DRFS of both luminal subtypes was significantly lower compared with their counterparts in node-negative disease (luminal A, HR = 3.29 and luminal B, HR = 2.26, $P < 0.0001$ for both comparisons). Regardless of nodal status, both luminal subtypes had continued risk of relapse after 5 years; even the lowest risk node-negative luminal A subtype had 5-year DRFS of 96% that dropped to 91% by 8.5 years. A tendency for worse outcomes was also observed in node-positive HER2-enriched tumors compared with node-negative HER2-enriched tumors (HR = 1.91, $P = 0.099$).

genomic relationships and biological significance

For comparisons across different predictors, the combined dataset was confined to the 594 samples/tumor represented by Affymetrix microarray data. We first compared the gene overlap between any two signatures and found that ≤25% of the genes were shared between signatures (supplemental Table S2, available at *Annals of Oncology* online), except for 9 and 11 genes of the GHI signature ($n = 21$) that were present

Table 1. Clinicopathological characteristics of the combined microarray and qRT-PCR patient dataset

	Symmans et al. [15]	%	Loi et al. [16, 17]	%	Zhang et al. [18]	%	Pawitan et al. [19]	%	Nielsen et al. [23]	%	Total	%
Dataset	MDACC298		LOI327		ZHANG136		PAWI159		NIELSEN			
GSE series	GSE17705	—	GSE6532	—	GSE12093	—	GSE1456	—	—	—	—	—
Patients[a]	298	100%	73	22%	136	100%	87	55%	786	100%	1380	—
Node-negative[b]	175	61%	20	29%	136	100%	57	69%	222	30%	610	47%
Node-positive[b]	112	39%	50	71%	0	0%	26	31%	511	70%	699	53%
PAM50 subtypes[c]												
Luminal A	132	44%	34	47%	66	49%	40	46%	372	47%	644	47%
Luminal B	100	34%	19	26%	48	35%	31	36%	329	42%	527	38%
HER2-enriched	16	5%	10	14%	5	4%	5	6%	64	8%	100	7%
Basal-like	8	3%	4	5%	4	3%	4	5%	5	1%	25	2%
Normal-like	42	14%	6	8%	13	10%	7	8%	16	2%	84	6%

[a]Only patients with ER+ disease treated with tamoxifen-only were selected from these datasets. In GSE6532, 103 samples have been removed since they overlap with GSE17705.

[b]GSE17705, GSE1456, GSE6532 and Nielsen et al. [23] have 11, 4, 3 and 53 patients without node status, respectively (total $n = 71$).

[c]Subtype data in Nielsen et al. were obtained by the qRT-PCR PAM50 assay.

qRT-PCR, quantitative RT-PCR.

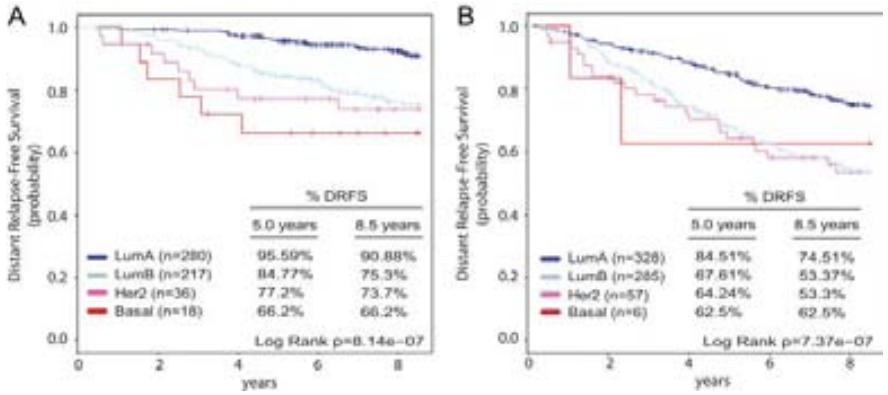

Figure 1. Kaplan–Meier DRFS analysis of intrinsic subtype as determined by PAM50 gene expression measurement (quantitative reverse transcription–PCR and microarray-based) from women with (A) node-negative and (B) node-positive invasive breast carcinoma, treated with adjuvant tamoxifen only. The number of patients and the estimated DRFS at 8.5 years in each group are shown beside each curve's description. DRFS, distant relapse-free survival.

in the IE-IIE and PAM50, respectively, and 15 genes of the IE-IIE signature that were present in PAM50. In spite of relatively little gene overlap, all predictors were significantly correlated (Pearson correlation range 0.36–0.79; $P < 0.0001$ for each comparison), with PAM50-RORS, IE-IIE and GHI showing the highest correlation between them (>0.72, $P < 0.0001$, Pearson correlation; supplemental Table S2, available at *Annals of Oncology* online).

The observed correlations suggested that most predictors are tracking tumors with similar biology. To further explore this hypothesis, we evaluated the scores of each signature as a continuous variable and as group categories across the four major intrinsic subtypes (as defined by the PAM50 assay [10]). As expected, each predictor discriminated luminal A tumors from the luminal B subtype and from the rest of the subtypes [$P < 0.0001$, Student's *t*-test (supplemental Figure S3 and Table S3, available at *Annals of Oncology* online)]. The high hormonal sensitivity groups (SET-high and IE-like) and low risk of recurrence groups (PAM50-RORS-low, PAM50-RORP-low, GHI-low, ROT76-good and NKI-good) were largely composed of luminal A tumors (>71%–100%).

survival analyses within node-negative and node-positive disease

Univariate DRFS analyses revealed that each signature, evaluated as a continuous variable or as group categories, was highly prognostic in patients with node-negative disease (supplemental Figure S4 and Table S4, available at *Annals of Oncology* online). As expected, Kaplan–Meier survival analyses showed highly significant differences in DRFS across the groups predicted to have good or intermediate or poor prognosis (PAM50-RORS, PAM50-RORP, GHI, ROT76 and NKI70) or the groups predicted to have high or intermediate versus low expression of ER-regulated genes (SET and IE-IIE). Importantly, all predictors identified groups of node-negative

Table 2. Low-risk group comparison among signatures

	Node-negative			Node-positive		
	Low-risk group			Low-risk group		
	N	% of luminal A	DRFS at 8.5 years	*N*	% of luminal A	DRFS at 8.5 years
RORP (PAM50)	82 (24%)	100%	94%	38 (22%)	100%	80%
RORS (PAM50)	140 (41%)	100%	90%	63 (37%)	100%	74%
PROLIF[a] (PAM50)	72 (22%)	100%	93%	33 (19%)	100%	82%
GHI	47 (14%)	94%	95%	14 (8%)	93%	81%
ROT76[b]	164 (48%)	85%	92%	81 (47%)	77%	76%
IE-IIE[b]	235 (69%)	72%	88%	100 (58%)	71%	69%
NKI70[b]	136 (40%)	78%	91%	53 (31%)	79%	76%
SET	26 (8%)	81%	96%	21 (12%)	91%	89%
RORP (PAM50)[c]	116 (21%)	100%	96%	116 (17%)	100%	84%
RORS (PAM50)[c]	197 (36%)	100%	91%	197 (29%)	100%	79%
PROLIF (PAM50)[c]	142 (26%)	99%	95%	166 (24%)	99%	80%

[a]Since proliferation (PROLIF) index does not have previously defined cut-offs, patients in the low-risk group are the ones with the lowest quartile expression.
[b]ROT76, IE-IIE and NKI70 signatures have two risk categories.
[c]qRT–PCR PAM50 data from the Nielsen series have been included. *N*, number of patients in the low-risk group and the percentage from the total number of patients based on node status.
DRFS, distant relapse-free survival.

patients with 93.7%–97.9% and 88.4%–96.2% DRFS at 5.0 and 8.5 years, respectively, although the number of patients in each group differed (Table 2); when limited to the combined microarray dataset and across the predictors with three risk categories (GHI, SET, PAM50-RORS and PAM50-RORP), the PAM50-RORS identified the largest number of low-risk patients ($n = 140$, 41%), followed by PAM50-RORP ($n = 82$, 24%), GHI ($n = 47$, 14%) and SET ($n = 27$%). Inclusion of the 786 ER+ patient qRT-PCR PAM50 Nielsen series data confirmed that both PAM50-RORP and PAM50-RORS identify 21%–36% of all node-negative patients ($n = 551$) as low risk [or alternatively they identify 41%–70% of all node-negative luminal A tumors ($n = 280$) as low risk], and the PAM50-RORP-low and PAM50-RORS-low groups showed a DRFS at 8.5 years of 96.09% and 91.21%, respectively (Table 2 and supplemental Figure S5, available at *Annals of Oncology* online).

In node-positive disease, univariate DRFS analyses revealed that most signatures were barely significant when evaluated as continuous variables (supplemental Figure S6 and Table S4, available at *Annals of Oncology* online). When evaluated as group categories, low risk of relapse or high expression of ER-regulated gene groups showed either no statistical significance or borderline significance in terms of DRFS compared with the predicted poor prognostic or low expressers of ER-regulated gene groups. More importantly, no predictor identified a clear node-positive group of patients treated with tamoxifen alone with a DRFS at 8.5 years >90%. Although these results could be related to the sample size, data for PAM50-RORS and PAM50-RORP in node-positive disease confirmed this finding when the qRT-PCR PAM50 Nielsen series was included for a total of 676 patients (supplemental Figure S5, available at *Annals of Oncology* online). Finally, similar to node-negative disease, the predicted low-risk outcome groups in node-positive disease were predominantly comprised of luminal A tumors (71%–100%; Table 2).

prognostic prediction performance

C-index values were calculated to estimate the performance of each genomic signature for predicting outcome (Figure 2). The C-index is a measure of the probability of concordance between the predicted and the observed survival, ranging from 0.5 (random) to 1 (perfect). Although no clear cut-off value has been defined, values >0.70 are indicative of good prediction accuracy [25]. In node-negative disease, the vast majority of signatures showed similar predictive abilities (mean C-index range of 0.70–0.73), except PAM50-PROLIF index (mean C-index of 0.69) and NKI70 (mean C-index of 0.64). Conversely, in node-positive disease, all predictors carried out worse than in node-negative (mean C-index range of 0.56–0.63).

Despite comparable prognostic performance of these signatures and high correlation values among them, we observed that these signatures generally retained their prognostic significance independent of each other when testing two signatures at a time in multivariate analyses (Table 3). Thus, we sought to determine if we could improve prognostic performance by integrating information from all signatures into a single model; we determined that the combined model was better at predicting outcome than individual signatures in node-negative disease (Figure 2A) but failed in node-positive disease (Figure 2B). However, the absolute increase in performance of the combined model within node-negative disease was modest (range 0.02–0.11).

prognostic predictions within the intrinsic subtypes

We explored the predictive ability of each signature within the predominant luminal A and B subtypes. In node-negative luminal A disease ($n = 185$), ROT76 and SET (Figure 3A) were found to be prognostic in univariate analyses, and patients with the low-risk group showed a DRFS at 8.5 years of 94%–96% (supplemental Table S5, available at *Annals of Oncology* online). When limited to the microarray dataset, the PAM50-

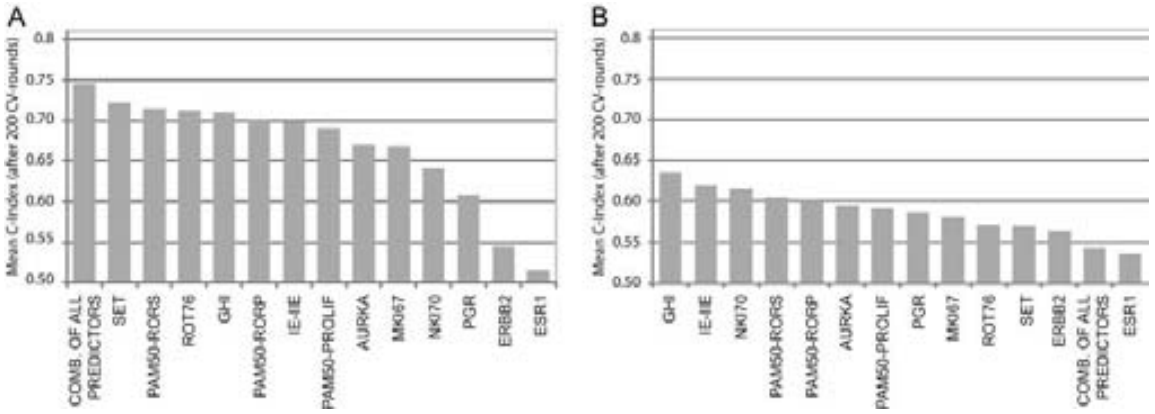

Figure 2. Comparison of prognostic classifiers and single genes in (A) node-negative and (B) node-positive subjects. The C-index is used to compare accuracy of the prognostic classifiers and single genes. Signatures have been ranked ordered from highest to lowest mean C-index. In node-negative disease, the C-index of the combined model was superior to the C-index of each individual signature in at least 75% of the 200 total estimations.

Table 3. Multivariate Cox proportional hazards analyses of distant relapse-free survival among predictors[a]

		Adjusted on the following predictor							
		PAM50-RORP	PAM50-RORS	PAM50-PROLIF	GHI	ROT76	IE-IIE	NKI70	SET
Predictor χ^2 statistic and *P* value	PAM50-RORP		10.6; 0.005	7.2; 0.027	11.5; 0.003	9.6; 0.008	10.45; 0.005	16.2; <0.001	17.4; <0.001
	PAM50-RORS	0.0; 0.990		1.0; 0.617	5.4; 0.067	2.9; 0.240	3.1; 0.220	7.1; 0.029	9.2; 0.012
	PAM50-PROLIF	0.2; 0.890	4.6; 0.099		7.6; 0.023	4.6; 0.100	5.8; 0.056	10.7; 0.005	13; 0.002
	GHI	9.1; 0.010	13.6; 0.001	12.2; 0.002		13.5; 0.001	13.4; 0.001	14.4; <0.001	20.0; <0.001
	ROT76	4.3; 0.031	8.29; 0.004	6.4; 0.012	10.7; 0.001		10.4; 0.001	13.0; <0.001	11.0; 0.0013
	IE-IIE	3.2; 0.072	6.4; 0.011	5.5; 0.019	8.6; 0.003	8.4; 0.004		9.2; 0.002	12.0; 0.001
	NKI70	5.7; 0.022	7.3; 0.007	7.24; 0.013	9.2; 0.002	7.8; 0.005	6.1; 0.014		14.0; <0.001
	SET	6.6; 0.042	9.0; 0.011	8.7; 0.012	13.6; 0.001	9.7; 0.008	8.5; 0.015	13.6; 0.001	

[a]Each square denotes the change in the likelihood ratio statistic (χ^2) of the signature in each row and its *P* value when conditioned on a signature in the column.

RORS and PAM50-RORP were trending toward significance (supplemental Table S5, available at *Annals of Oncology* online) and both were significant when the Nielsen series was included for a total of 280 luminal A patients (supplemental Table S5, available at *Annals of Oncology* online and PAM50-RORP in Figure 3B).

In node-positive luminal A disease ($n = 81$) on the microarray dataset, GHI, NKI70 and IE-IIE were prognostic when evaluated as a continuous variable, and the combined low and intermediate risk GHI groups identified a group of significantly low-risk node-positive luminal A tumors ($n = 30$, 37%) with an outstanding DRFS at 8.5 years (96%, $P < 0.01$; Figure 3C). When we included the qRT-PCR PAM50 Nielsen series dataset ($n = 326$), PAM50-RORS and PAM50-RORP were found prognostic as a continuous variable and as group categories, with the low-risk PAM50-RORP group achieving a DRFS at 8.5 years of 84.02% ($P < 0.01$; Figure 3D).

Within the luminal B subtype ($n = 120$), the vast majority of signatures were found to be prognostic when evaluated as a continuous variable in node-negative disease (supplemental Table S6, available at *Annals of Oncology* online); however, no statistically significant group of patients with >90% DRFS at 8.5 years was identified by any of the predictors (supplemental Table S6, available at *Annals of Oncology* online); similar findings were obtained when we included the qRT-PCR PAM50 Nielsen series dataset. Finally, no significant prognostic ability was found within node-positive luminal B tumors, with ($n = 285$) or without ($n = 70$) the Nielsen series, respectively (supplemental Table S6, available at *Annals of Oncology* online).

discussion

Our data indicates that (i) clinically used signatures and ER-regulated gene signatures are tracking tumors with similar underlying biology (luminal A versus not) and show significant agreement in outcome predictions; (ii) the performance of these signatures is most relevant in node-negative disease; and (iii) some single genomic signatures can perform nearly as well as a combination of two or more signatures, although a combination of multiple signatures was statistically the best. Importantly, this is the first report to show that groups of patients with >95% DRFS at 8.5 years might only be consistently identified within node-negative and luminal A disease. Alternatively, for patients with luminal B cancer treated only with tamoxifen, additional therapies should be offered, which, as of today, would suggest chemotherapy.

These results also demonstrate that most of the signatures evaluated in this study can provide similar outcome predictions, although significant differences across predictors

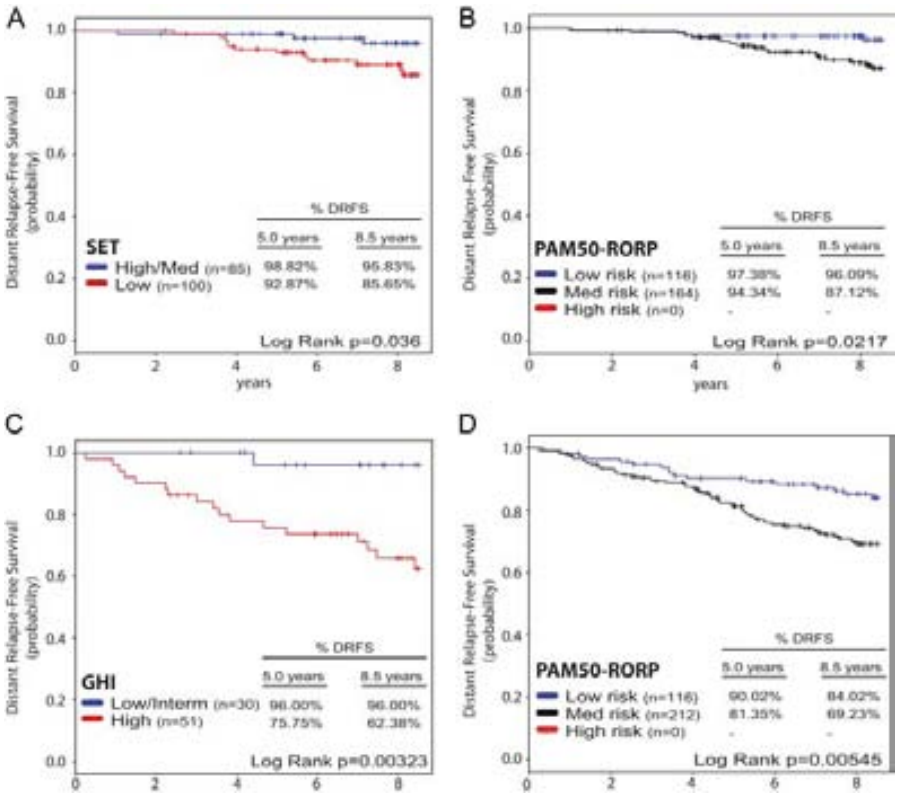

Figure 3. Kaplan–Meier DRFS analysis of selected gene signatures within luminal A disease treated with adjuvant tamoxifen only. (A) SET index within node-negative luminal A tumors; (B) PAM50-RORP within node-negative luminal A tumors (Nielsen series included); (C) GHI within node-positive luminal A tumors; (D) PAM50-RORP within node-positive luminal A tumors (Nielsen series included). The complete survival analyses can be found in supplemental Tables S5 and S6, available at *Annals of Oncology* online. DRFS, distant relapse-free survival.

are present. This result is harmonious with our previous observation of concordance between intrinsic subtypes, NKI70 and GHI in a cohort of heterogeneously treated ER+ and ER− breast cancer patients [12]. Importantly, here, we show that these and other signatures are tracking ER+ tumors with a similar biology. Indeed, the vast majority of ER+ tumors identified here as having either basal-like, HER2-enriched or luminal B subtypes were correctly classified by the other signatures as having a poor prognosis. On the other hand, luminal A tumors were mostly identified as having good outcome and showing high expression of ER-regulated signatures. Interestingly, a recent neoadjuvant aromatase inhibitor clinical trial reported that the luminal A subtype benefits the most from endocrine therapy [26].

The performance of each predictor in node-positive disease was significantly worse when compared with node-negative disease, and almost no group of patients with node positivity had a DRFS >90% at 8.5 years by any predictor; the only exceptions being GHI within luminal A disease. In two previously published node-positive ER+ cohorts receiving adjuvant endocrine treatment only (TransATAC and SWOG-8814), the 9-year DRFS and 10-year disease-free survival estimates were 83% and 60% for the low-risk groups of the GHI, respectively [27, 28]. A plausible biological explanation is that in advanced luminal A primaries, a small percentage of cells within the bulk of the tumor have already metastasized and/or acquired endocrine resistance. Indeed, the presence of these subclones is supported by data from a neoadjuvant endocrine trial [29]. However, within node-positive luminal A tumors, some patients with the low and intermediate risk score of GHI had a DRFS at 8.5 years >90%. Hence, future studies are warranted to determine if these or other predictors can identify, within the luminal A subtype, a group of node-positive patients whose survival with endocrine therapy could preclude the administration of adjuvant chemotherapy. The MINDACT [6] trial, which has completed accrual, and the RxPONDER trial (NCT01272037) will address this issue, particularly for patients with one to three positive lymph nodes.

Multivariate analyses including two predictors at a time revealed that, in most cases, many of these correlated predictors, in particular the PAM50-RORP, GHI, NKI70 and SET, remained statistically independent of each other (Table 3). This finding suggests that these predictors are not the same. In fact, at the individual level, the risk group assignment concordance among these predictors was found to be 36% for PAM50-RORP versus GHI, 54% for PAM50-RORP (low/med versus high) versus NKI70 and 74% for GHI (low/intermediate versus high) versus NKI70. Cohen's kappa coefficients between risk group assignments were also indicative of slight to fair agreement (range 0.11–0.42) [30, 31]. The clinical relevance of this finding is currently unknown. However, a plausible explanation is that these signatures might be tracking different poor outcome luminal/ER+ subtypes; support for this

heterogeneity comes from Parker et al. [10], where five statistically significant groups of luminal tumors were identified. Nonetheless, when we built a model here using all predictors, we only observed modest improvements in performance. This finding suggests that gene expression profiling may be reaching its maximum prognostic power.

There are several important caveats to our analyses that must be recognized and always kept in mind when interpreting 'across platform' genomic studies. First, although we strove to implement each predictor as published, signatures developed on platforms other than the Affymetrix U133A were suboptimally implemented. This is because when taking a predictor from one technology and applying it to another platform, different oligonucleotide probes/sequences are used to represent each gene (and thus may not behave identically), and each technology has unique normalization methods. Second, changing platforms/technologies almost always causes a loss of genes (see supplemental Table S1, available at *Annals of Oncology* online), and this loss was significantly present for PAM50 (6/50) and NKI70 (12/60), which likely explains the observed lower performance of this predictor with respect to others. Nonetheless, many of the across platform evaluated predictors carried out well including the PAM50-ROR and GHI; the survival outcomes of the GHI low-risk group within node-negative disease was highly concordant to previous publications [32] despite that the absolute survival rates are highly dataset dependent. Finally, we could not compare the prognostic ability of these signatures versus standard clinicopathological variables since these variables were not available from most microarray datasets. This highlights the need for centralized collection of clinical and pathology data in all genomic studies.

To conclude independently derived genomic predictors of breast cancer recurrence perform similarly and are tracking tumors with similar biology. However, most predictors were statistically independent from the others and thus, these should not be considered to be interchangeable assays. From a clinical perspective, adding genomic signatures together provided modest improvements in outcome prediction, but may not be practical given cost.

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disclosure

CMP, PSB, TON and MJE are equity stock holders of University Genomics and BioClassifier LLC. CMP, PSB, MCUC, TON, MJE and JSP have filed a patent on the PAM50 assay. AP, CF, LDM, JB, SKLC and LAC have declared no conflicts of interest.

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Explanatory factors of sexual function in sexual minority women breast cancer survivors

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Background: The sexual function of sexual minority women (women with female partners) who are breast cancer survivors is mostly unknown. Our objective is to identify explanatory factors of sexual function among sexual minority women with breast cancer and compare them with a control sample of sexual minority women without cancer.
Patients and methods: Using a conceptual framework that has previously been applied to heterosexual breast cancer survivors, we assessed the relationship of each explanatory factor to sexual function in sexual minority women. Using generalized estimating equations, we identified explanatory factors of sexual function and identified differences by case and control status.
Results: Self-perception of greater sexual attractiveness and worse urogenital menopausal symptoms explain 44% of sexual function, after controlling for case and control status. Focusing only on partnered women, 45% of sexual function was explained by greater sexual attractiveness, postmenopausal status, and greater dyadic cohesion.
Conclusions: All of the relevant explanatory factors for sexual function among sexual minority survivors are modifiable as has been suggested for heterosexual survivors. Sexual minority survivors differ from heterosexual survivors in that health-related quality of life is less important as an explanatory factor. These findings can guide adaptation of interventions for sexual minority survivors.
Key words: breast neoplasm, case–control study, female, homosexuality, sexual dysfunctions

introduction

Sexual dysfunction or difficulties remain a persistent concern of breast cancer survivors (BCS) [1–3]. Sexual dysfunction is common and distressing, affecting ~50% of BCS [3–5].

Depending on the dimension of sexual function (desire, arousal, orgasm, frequency of sexual activity) measured, the incidence of sexual dysfunction varies from 15% to a high of 64% [4, 5]. Broeckel et al. [6] demonstrated worse sexual functioning in long-term BCS compared with controls, including greater lack of sexual interest, inability to relax and enjoy sex, difficulty becoming aroused, and difficulty achieving orgasm. Study findings are inconsistent when sexual frequency is used as the measure of sexual functioning: Ganz et al. [4, 7] found no

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BRIEF REPORT

PAM50 assay and the three-gene model for identifying the major and clinically relevant molecular subtypes of breast cancer

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Abstract It has recently been proposed that a three-gene model (SCMGENE) that measures ESR1, ERBB2, and AURKA identifies the major breast cancer intrinsic subtypes and provides robust discrimination for clinical use in a manner very similar to a 50-gene subtype predictor (PAM50). However, the clinical relevance of both predictors was not fully explored, which is needed given that a ~30 % discordance rate between these two predictors was observed. Using the same datasets and subtype calls provided by Haibe-Kains and colleagues, we compared the SCMGENE assignments and the research-based PAM50 assignments in terms of their ability to (1) predict patient outcome, (2) predict pathological complete response (pCR) after anthracycline/taxane-based chemotherapy, and (3) capture the main biological diversity displayed by all genes from a microarray. In terms of survival predictions, both assays provided independent prognostic information from each other and beyond the data provided by standard clinical–pathological variables; however, the amount of prognostic information was found to be significantly greater with the PAM50 assay than the SCMGENE assay. In terms of chemotherapy response, the PAM50 assay was the only assay to provide independent predictive information of pCR in multivariate models. Finally, compared to the SCMGENE predictor, the PAM50 assay explained a significantly greater amount of gene expression diversity as captured by the two main principal components of the breast cancer microarray data. Our results show that classification of the major and clinically relevant molecular subtypes of breast cancer are best captured using larger gene panels.

Keywords Breast cancer · Microarrays · PAM50 · Prognosis · Gene expression

Introduction

Over the years, global gene expression analyses have identified at least four intrinsic subtypes of breast cancer (Luminal A, Luminal B, HER2-enriched, and Basal-like) and a normal-like group with significant differences in terms of their risk factors, incidence, baseline prognoses and responses to systemic therapies [1–4]. In 2009, we reported a clinically applicable gene expression-based predictor that robustly identifies these main intrinsic subtypes by quantitative measurement of 50 genes (i.e., PAM50) [1]. Identification of these molecular subtypes using pathology-based surrogate definitions based upon hormone receptors (HRs), HER2 and Ki-67 expressions has been adopted by the 2011 St. Gallen Consensus Conference for treatment decision-making in early breast cancer [5], however, controversy exists as to whether these complex

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molecular subtypes can be effectively captured using four or less biomarkers.

Recently, Haibe-Kains et al. [6] reported a mRNA expression predictor that classifies tumors into four molecular entities (ER+/HER2−/Low Proliferative, ER+/HER2−/High Proliferative, HER2+ and ER−/HER2−) by quantitative measurement of three genes (ESR1, ERBB2 and AURKA). Similar to the PAM50 subtype predictions, the molecular entities identified by the SCMGENE predictor were found significantly associated with survival outcome [6]. However, a direct head-to-head comparison between both predictors was not performed despite that fact that the concordance (i.e., κ score) between these two predictors was 0.59 (0.58–0.61), which is considered moderate agreement and similar to the κ scores obtained when histological grade is evaluated by two independent observers [7].

In this study, we compared the SCMGENE assignments and the research-based PAM50 assignments in terms of their ability to (1) predict patient outcome, (2) predict pathological complete response (pCR) after anthracycline/taxane-based chemotherapy, and (3) capture the main biological diversity displayed by all genes from a microarray.

Materials and methods

Clinical and gene expression data

We used the clinical (Supplemental file: jnci-JNCI-11-0924-s02.csv) and gene expression data (http://www.compbio.dfci.harvard.edu/pubs/sbtpaper/data.zip) as provided by Haibe-Kains et al. [6]. For survival predictions, we used distant metastasis-free survival as the endpoint since it provides the largest number of patients that can be evaluated across 13 datasets (CAL [8], EMC2 [9], DFHCC [10], MAINZ [11], MDA5 [12], MSK [13], NKI [14], TAM [15], TRANSBIG [16], UCSF [17], UNT [18], VDX [19] and VDX3 [20]). None of the datasets (or samples) used for survival (or response prediction) were used to derive the SCMGENE or the PAM50 subtype predictor.

To compare chemotherapy response data, we used the clinical data of one of the datasets (MAQC2 [GSE20194] [21]) evaluated by Haibe-Kains et al. [6], which is composed of 230 pre-treatment samples with annotated response data (pCR vs. residual disease [RD]) after neo-adjuvant anthracycline/taxane-based chemotherapy. Samples that received trastuzumab were excluded.

Combined microarray dataset

Eighteen Affymetrix and Agilent-based datasets (CAL [8], DFHCC [10], DUKE [22], EORTC10994 [23], EXPO [24], KOO [25], MAINZ [11], MAQC2 [21], MDA4 [26], MSK [13], NKI [14], PNC [27], STK [28], TRANSBIG [16], UNC337 [29], UNT [18], UPP [30] and VDX [19]) as provided in Haibe-Kains et al. [6] and with an appropriate distribution of ER+ (50–90 %, as defined by IHC) versus ER− tumors were combined into a single gene expression matrix. Probes mapping to the same gene (Entrez ID as defined by the manufacturer) were averaged to generate independent expression estimates. In each cohort, genes were median centered and standardized to zero mean and unit variance.

Statistical analyses

Distant metastasis-free survival univariate and multivariate analysis were calculated using a Cox proportional regression model. Likelihood ratio statistics of subtypes defined by the PAM50 or the SCMGENE predictors were also evaluated after accounting for clinical–pathological variables (age at diagnosis, nodal status, and tumor size) and type of systemic adjuvant treatment (chemotherapy, endocrine, and none). Models were first conditioned on one predictor and the clinical–pathological variables, and then the significance of the other was tested. Chemotherapy response (pCR vs. RD) predictions of each variable were evaluated using univariate and multivariate logistic regression analyses. Finally, R^2 values of each predictor (SCMGENE or PAM50) for each principal component (PC) were calculated using a simple linear regression model. All statistical computations were performed in R v.2.8.1 (http://www.cran.r-project.org).

Results

Outcome prediction

To compare the ability of the SCMGENE and PAM50 assays to predict patient outcome, we performed Cox proportional hazard regression analyses using the entire combined dataset as provided by Haibe-Kains et al. [6]. In the multivariate model (MVA), both predictors were found significantly associated with distant metastasis-free survival (Table 1) and the Luminals A and B segregation of the PAM50 assay was found significantly associated with outcome, whereas the ER+/HER2−/Low Proliferative and ER+/HER2−/High Proliferative segregation of the SCMGENE predictor was not. Conversely, distant metastasis-free survival differences of the ER−/HER2− versus the ER+/HER2−/Low Proliferative groups were found significant, whereas the Basal-like versus Luminal A segregation was not.

To compare the amount of independent prognostic information provided by each predictor, we estimated the likelihood ratio statistic of each predictor in a model that already included clinical–pathological variables (age,

Table 1 Distant metastasis-free survival Cox proportional hazards models of primary breast cancer patients

Variables	Univariate analysis				Multivariate analysis			
	HR	Lower 95 %	Upper 95 %	*p* Value	HR	Lower 95 %	Upper 95 %	*p* Value
Age (cont. variable)	0.989	0.983	0.996	0.003	0.996	0.988	1.003	0.257
Node status	1.176	0.851	0.992	0.063	1.695	1.315	2.184	<0.001
Tumor size T2–T4 versus T0–T1	1.305	1.104	1.541	0.002	1.242	1.042	1.480	0.015
Treatment (yes vs. no)	0.973	0.845	1.121	0.707	0.547	0.428	0.700	<0.001
PAM50								
Luminal A	1.0	–	–	–	1.0	–	–	–
Luminal B	1.797	1.503	2.149	<0.001	2.041	1.578	2.641	<0.001
HER2-E	2.677	2.120	3.380	<0.001	1.648	1.073	2.530	0.023
Basal-like	2.144	1.737	2.647	<0.001	1.312	0.812	2.121	0.268
Normal-like	1.073	0.670	1.718	0.769	1.024	0.572	1.835	0.936
Three-gene signature								
ER+/HER2−/Low Prolif	1.0	–	–	–	1.0	–	–	–
ER+/HER2−/High Prolif	1.852	1.531	2.241	<0.001	1.153	0.882	1.508	0.297
HER2+	2.785	2.196	3.533	<0.001	1.588	1.053	2.395	0.028
ER−/HER2−	2.536	2.041	3.150	<0.001	1.762	1.095	2.835	0.020

HER2-E HER2-enriched, *Prolif* proliferation, *HR* hazard ratio

tumor size, treatment and nodal status) and the other predictor. The results revealed that the PAM50 subtypes provide a larger amount of independent prognostic information than the SCMGENE subtypes when using the entire cohort of heterogeneously treated patients (Fig. 1A, B). Similar results were observed when using the subset of patients that did not receive adjuvant systemic therapy (Fig. 1C, D), and in the subset of patients with HR+ tumors that received adjuvant tamoxifen-only (Fig. 1E, F).

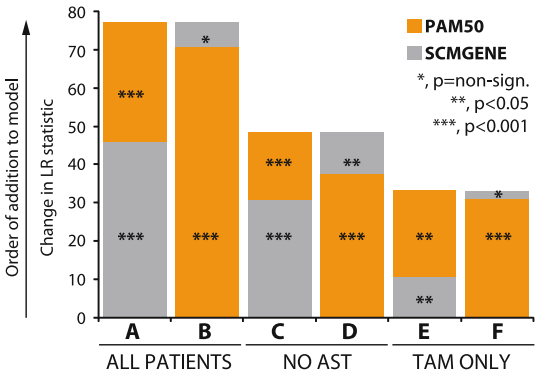

Fig. 1 Distant metastasis-free survival likelihood ratio statistics of subtypes defined by the PAM50 or the SCMGENE predictors, after accounting for clinical–pathological variables (age at diagnosis, nodal status, treatment and tumor size). Models were first conditioned on one predictor and the clinical–pathological variables, and then the significance of the other was tested. (*A–B*) Entire combined dataset ($n = 2{,}008$), (*C–D*) subset of patients that did not receive adjuvant systemic therapy ($n = 994$), (*E–F*) subset of patients with HR+ tumors that received adjuvant tamoxifen-only ($n = 491$). Similar results are obtained if a term for dataset is included in the model

Chemotherapy response prediction

To compare the ability of the PAM50 and SCMGENE assays to predict response to chemotherapy, we evaluated the MAQC2 (GSE20194) [21] dataset included in Haibe-Kains et al. [6] analyses. This cohort is composed of 226 pre-treatment samples with annotated response data (pCR vs. RD) after neoadjuvant anthracycline/taxane-based chemotherapy (without trastuzumab for HER2+ disease). As shown in Table 2, although both assays predicted response in univariate analysis, the PAM50 assay was the only one to provide independent predictive information in the MVA model.

Of note, the association of the PAM50 subtype with response was strengthened when PAM50 subtyping of the MAQC2 dataset was performed after median centering the PAM50 genes/rows (Supplemental Table 1). In fact, we and others have previously proposed median gene centering to minimize technical bias and allow the correct identification of the PAM50 intrinsic subtypes when appropriate representation of ER−, ER+, and HER2+ samples is available [31, 32]. Median gene centering of the UNC337 dataset before PAM50 or SCMGENE predictions also improved the survival classifications (Supplemental Fig. 1).

Capturing the main biological diversity

Finally, to compare both predictors in terms of their ability to capture the main biological diversity displayed by all genes in a breast cancer microarray, we first combined 18 datasets evaluated by Haibe-Kains et al. [6] and identified the two

Table 2 pCR logistic regression models of the MAQC2 (GSE20194) [21] neoadjuvant breast cancer dataset

Variables	*N*	pCR rate (%)	Univariate analysis				Multivariate analysis			
			OR	Lower 95 %	Upper 95 %	*p* Value	OR	Lower 95 %	Upper 95 %	*p* Value
Age (cont. variable)	–	–	1.0	0.95	1.01	0.169	–	–	–	–
Tumor size										
T0–T1	23	35	1.0	–	–	–	1.0	–	–	–
T2–T4	203	19	2.3	0.92	5.86	0.076	0.4	0.13	1.23	0.111
PAM50										
Luminal A	66	3	1.0	–	–	–	1.0	–	–	–
Luminal B	66	9	3.2	0.62	16.47	0.164	5.2	0.68	37.97	0.108
HER2-E	28	46	23.5	5.25	105.36	<0.001	12.5	1.46	145.68	0.030
Basal-like	59	42	27.7	5.65	136.18	<0.001	25.3	2.64	255.95	0.005
Normal-like	7	0	0.0	0.00	–	0.988	0.0	0.00	–	0.988
Three-gene signature										
ER+/HER2−/Low Prolif	52	4	1.0	–	–	–	1.0	–	–	–
ER+/HER2−/High Prolif	85	8	2.2	0.45	11.23	0.325	0.6	0.08	4.62	0.633
HER2+	24	50	25.0	4.93	126.80	<0.001	3.9	0.34	46.46	0.275
ER−/HER2−	65	38	15.6	3.49	69.93	<0.001	0.9	0.09	9.97	0.954

HER2-E HER2-enriched, *Prolif* proliferation, *OR* odds ratio

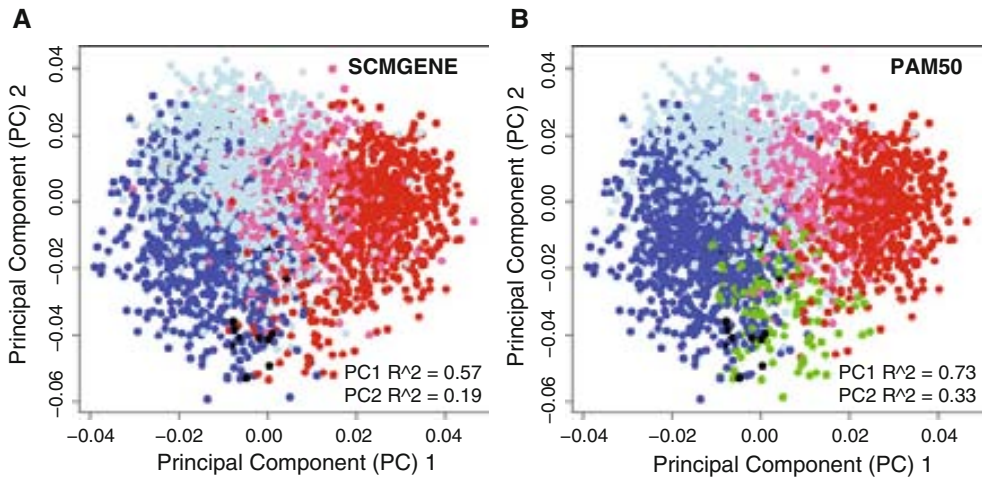

Fig. 2 PC1 and PC2 loading plots of 3,316 samples using 18 Affymetrix and Agilent-based datasets taken from Haibe-Kains et al. [6]. Samples *colored* based on the **a** SCMGENE calls, or **b** PAM50 subtype calls. PC1 and PC2 R^2 values obtained from simple linear regression models are shown. Only datasets with >50 % and <90 % ER+ tumors were included in this analysis. *Blue* Luminal A or ER+/HER2−/Low Proliferative, *light blue* Luminal B or ER+/HER2−/High Proliferative, *pink* HER2-enriched or HER2+, *red* Basal-like or ER−/HER2−, *green* normal-like, *black* normal breast samples (only present in the UNC337 dataset [29]). For the UNC337 dataset, we colored samples based on the subtype calls obtained after median centering as shown in Supplemental Fig. 1

main *p*rincipal *c*omponents (PC1 and PC2). Compared to the SCMGENE subtypes, the PAM50 subtypes explained substantially more variation in gene expression for both PC1 and PC2 (Fig. 2a, b), with these components being especially prominent for the separation of the Luminal A (or ER+/HER2−/Low Proliferative) and Luminal B (or ER+/HER2−/High Proliferative) subtypes. To confirm these findings, we also evaluated all PCs in each normalized dataset provided by Haibe-Kains et al. [6] and observed that among 483 PCs significantly explained by either one of the predictors, the PAM50 explained 2.27 times more independent variation in expression than the SCMGENE assay.

Discussion

Our results presented here, using the same data provided by Haibe-Kains et al. [6], suggest that (1) the SCMGENE and the PAM50 predictors should not be considered the same in terms of outcome prediction; (2) both provide independent

prognostic information; (3) the amount of prognostic information provided by the PAM50 predictor is greater than the information provided by the SCMGENE predictor; and (4) the PAM50 assay is the only independent predictor of neoadjuvant chemotherapy response.

A potential explanation of our findings is that the biological diversity of breast cancer is better captured using the quantitative measurement of the 50 PAM50 gene set compared to the 3 genes of the SCMGENE assay. This finding is further supported by our previous data during the PAM50 assay development, where the minimum number of genes required to identify the intrinsic molecular subtypes, as defined by subtype classifications based upon the ~1,900 intrinsic gene list with a 93 % accuracy, was the final selected 50 genes [1]. In fact, gene sets with less than 50 genes showed significantly worse accuracies, particularly for tumors of the Luminal B and HER2-enriched subtypes (Supplemental Fig. 2). Importantly, only 33.3 % (12/36) of all microarray datasets evaluated in Haibe-Kains et al. [6] had all the PAM50 genes available, whereas 100 % of the datasets had all three genes of the SCMGENE assay, thus highlighting another caveat of this study.

In total, these analyses show that a combination of ER, HER2, and a single proliferation biomarker (i.e., AURKA) is prognostic, but is suboptimal to capture the biological diversity of breast cancers, which has similar implications for the capture of this biological diversity using IHC-based methods. Although a head-to-head comparison of both assays in terms of their clinical utility might be warranted in the future, our results suggest that classification of the major and clinically relevant molecular subtypes is better achieved using larger gene sets that capture a greater proportion of the biological diversity of breast cancers.

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Conflict of interest C. M. P. is a stock holder of BioClassifier LLC. C. M. P. and J. S. P. have filed a patent on the PAM50 assay. A. P. and C. F. have declared no conflicts of interest.

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ORIGINAL REPORT

Prognostic Significance of Progesterone Receptor–Positive Tumor Cells Within Immunohistochemically Defined Luminal A Breast Cancer

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ABSTRACT

Purpose
Current immunohistochemical (IHC)-based definitions of luminal A and B breast cancers are imperfect when compared with multigene expression-based assays. In this study, we sought to improve the IHC subtyping by examining the pathologic and gene expression characteristics of genomically defined luminal A and B subtypes.

Patients and Methods
Gene expression and pathologic features were collected from primary tumors across five independent cohorts: British Columbia Cancer Agency (BCCA) tamoxifen-treated only, Grupo Español de Investigación en Cáncer de Mama 9906 trial, BCCA no systemic treatment cohort, PAM50 microarray training data set, and a combined publicly available microarray data set. Optimal cutoffs of percentage of progesterone receptor (PR) –positive tumor cells to predict survival were derived and independently tested. Multivariable Cox models were used to test the prognostic significance.

Results
Clinicopathologic comparisons among luminal A and B subtypes consistently identified higher rates of PR positivity, human epidermal growth factor receptor 2 (HER2) negativity, and histologic grade 1 in luminal A tumors. Quantitative PR gene and protein expression were also found to be significantly higher in luminal A tumors. An empiric cutoff of more than 20% of PR-positive tumor cells was statistically chosen and proved significant for predicting survival differences within IHC-defined luminal A tumors independently of endocrine therapy administration. Finally, no additional prognostic value within hormonal receptor (HR) –positive/HER2-negative disease was observed with the use of the IHC4 score when intrinsic IHC-based subtypes were used that included the more than 20% PR-positive tumor cells and vice versa.

Conclusion
Semiquantitative IHC expression of PR adds prognostic value within the current IHC-based luminal A definition by improving the identification of good outcome breast cancers. The new proposed IHC-based definition of luminal A tumors is HR positive/HER2 negative/Ki-67 less than 14%, and PR more than 20%.

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INTRODUCTION

Hormonal receptor (HR) –positive breast cancer is a clinically and biologically heterogeneous entity.[1-3] Studies based on gene expression profiling have identified at least two major groups of HR-positive tumors, known as the luminal A and B intrinsic subtypes of breast cancer. These two molecular entities have shown significant differences in baseline prognosis and sensitivity to cytotoxic therapies.[4-6]

Currently, a gene expression–based assay known as the PAM50 subtype predictor identifies the intrinsic molecular subtypes of breast cancer and provides a risk of relapse (ROR) score in a fashion similar to the Oncotype DX (Genomic Health, Redwood City, CA) recurrence score (RS).[4-6] These two assays provide valuable and independent prognostic information beyond standard clinicopathologic variables. However, standardized gene expression–based tests are not readily available in most of the world as a result of cost, assay turnaround times, and other logistic issues. Thus surrogate definitions of the intrinsic subtypes and/or risk of relapse groups developed using routine pathology and clinical parameters could be of great practical value.[7,8]

We have previously reported an immunohistochemical (IHC)-based surrogate definition of the luminal A (IHC-luminal A) and luminal B/human epidermal growth factor receptor 2 (HER2)-negative (IHC-luminal B/HER2-negative) subtypes based on the quantitative expression of the proliferation-related marker Ki-67 within HR-positive/HER2-negative disease.[9] This definition has now been adopted by the 2011 St Gallen Expert Consensus Panel Recommendation Guidelines for the systemic treatment of early breast cancer,[10] which recommend adjuvant endocrine therapy alone for patients with IHC-luminal A tumors and the addition of chemotherapy for patients with IHC-luminal B/HER2-negative tumors. Here we further refine the IHC-based definition of luminal A and B through the use of quantitative progesterone receptor (PR) expression.

PATIENTS AND METHODS

Patients, Samples, and Clinical Data

Multiple different and independent data sets were used to assess the significance of PR IHC results. Gene expression and/or clinicopathologic features were evaluated across five different data sets: (1) a combined genomic data set of nine publicly available microarray cohorts (GSE18229, GSE18864, GSE22219, GSE25066, GSE2990, GSE4922, GSE7390, GSE7849, and NKI295), (2) the PAM50 microarray-based subtype predictor training data set (PAM50-training, GSE10886),[5] (3) a British Columbia Cancer Agency (BCCA) tamoxifen-treated cohort (BCCA-tamoxifen),[6] (4) the Grupo Español de Investigación en Cáncer de Mama (GEICAM) 9906 trial,[11] and (5) the BCCA no adjuvant systemic therapy (AST) cohort (BCCA-no AST).[9] A detailed CONSORT diagram can be found in Appendix Table A1 (online only).

All patients from the BCCA-tamoxifen cohort[6] had early-stage HR-positive disease and received adjuvant treatment with tamoxifen only. In the GEICAM 9906 phase III trial cohort,[11] patients with node-positive disease were randomly assigned to adjuvant fluorouracil, epirubicin, and cyclophosphamide versus fluorouracil, epirubicin, and cyclophosphamide followed by weekly paclitaxel, and patients with HR-positive disease subsequently received adjuvant endocrine therapy. The BCCA-no AST cohort[9] includes "clinically low risk" patients with primary breast cancer diagnosed between 1986 and 1992 who did not receive adjuvant systemic therapy. Characteristics of both BCCA cohorts and the GEICAM 9906 cohort have been previously described.[6,9,11] From the PAM50-training cohort, we performed global and single gene expression analyses using only the prototypical samples of the luminal A and B subtype. Finally, the combined microarray data set included nine publicly available data sets of primary breast cancers with annotated clinicopathologic data.

PAM50 Intrinsic Subtyping

All tumors were assigned an intrinsic molecular subtypes of breast cancer (luminal A, luminal B, HER2-enriched, and basal-like) and the normal-like group using the PAM50 subtype predictor.[5,6] In the BCCA-tamoxifen and GEICAM 9906 cohorts,[11] PAM50 was determined using a quantitative reverse-transcriptase polymerase chain reaction–based assay.[5,6] In the GEICAM 9906 cohort, we evaluated the PAM50 ROR score based on subtype and proliferation (ROR-P) as previously described for the BCCA-tamoxifen cohort.[6] In each individual publicly available microarray cohort, we applied the PAM50 microarray-based algorithm[5] after data set to data set normalization based on median gene centering within each data set.

IHC-based subtyping was determined using the following definitions adopted by the 2011 St Gallen Consensus Panel[10]: IHC-luminal A (HR positive/HER2 negative/Ki-67 $<$ 14%), IHC-luminal B/HER2-negative (HR positive/HER2 negative/Ki-67 $>$ 14%), IHC-luminal B/HER2-positive (HR positive/HER2 positive), IHC-HER2+ (HR negative/HER2 positive), and triple-negative (HR negative/HER2 negative). Detailed IHC-based protocols for estrogen receptor (ER), PR, HER2, and Ki-67 determinations have been previously described[6,9,11,12] and are summarized in Appendix Table A2 (online only). All IHC-based tissue microarray images of both BCCA cohorts can be obtained via the Genetic Pathology Evaluation Centre TMA Viewer.[13]

IHC4 Score

A version of the IHC4 score was evaluated in HER2-negative disease using the reported formula.[8] However, instead of using the H-score reported in Cuzick et al[8] for estimating the semiquantitative expression of ER, we determined a general intensity score value of 0 to 3 and multiplied this value by the percentage of ER-positive tumor cells for a final ER score of 0 to 300.

Statistical Analysis

Significant differences in clinicopathologic features between groups were evaluated using either the χ^2 test or the t test. Estimates of survival were from the Kaplan-Meier curves and tests of differences by the log-rank test. Univariate and multivariate Cox models were used to test the independent prognostic significance of each variable. Over-represented biologic processes were identified with Expression Analysis Systematic Explorer (EASE).[14]

To identify an optimal cutoff of percentage of PR-positive tumor cells within IHC-luminal A tumors, we applied the penalized spline method on multivariable Cox regression analysis in the BCCA-tamoxifen cohort[6] (training data set), and the optimal cutoff to predict distant relapse–free survival (DRFS) was independently tested in the GEICAM 9906[11] and BCCA-no AST[9] cohorts.

To test the contribution of the IHC4 score, IHC-based subtyping and the PAM50 ROR-P score, all of these variables were tested in a prognostic model within HR-positive/HER2-negative disease. Here we estimated the log likelihood ratio statistic of each variable as an addition to a model containing the following clinical variables in the GEICAM 9906 cohort[11]: treatment arm, histologic grade, tumor stage, nodal status, and age. Finally, we estimated the log likelihood ratio statistic of each variable as an addition to a model containing clinical variables and one or two of the three variables being evaluated (IHC4 score, intrinsic IHC-based subtyping, and PAM50 ROR-P).

RESULTS

Gene and Protein Expression Differences Between Luminal A and B Tumors

To identify global and single gene expression differences, we performed a two-class significance analysis of microarrays between prototypical luminal A and B tumors from the PAM50-training cohort.[5] A total of 1,539 genes (348 upregulated and 1,191 downregulated) were found differentially expressed (false discovery rate $<$ 1%) between both subtypes (Appendix Fig A1, online only; Data Supplement). The upregulated gene list in luminal A tumors was found enriched for genes involved in cell differentiation (eg, Kruppel-like factor 4 and *jun* proto-oncogene) and cell adhesion (eg, vinculin and collagen, type XVI, α1) biologic processes. Conversely, the downregulated gene list in luminal A tumors (ie, genes highly expressed in luminal B tumors) was found enriched for genes involved in immune response (eg, interleukin 2 receptor α and *CD86*) and cell-cycle (eg, cyclin B1 and *RAD51*) biologic processes, which is indicative of the faster proliferation rates known to be part of luminal B tumors.

Among the relatively upregulated genes in luminal A tumors was the progesterone receptor gene (*PGR*), but not the estrogen receptor gene (*ESR1*). To further explore these findings, we evaluated the mRNA expression of *PGR* and *ESR1* in two independent studies in which PAM50 was performed using the quantitative reverse-transcriptase polymerase chain reaction platform (GEICAM 9906[11] and BCCA-tamoxifen[6]) and confirmed that *PGR*, but not *ESR1*, was found significantly upregulated in luminal A tumors compared with luminal B tumors (Figs 1A and 1B; $P < .001$, t test). Interestingly, *PGR* was found only weakly correlated (Pearson correlation coefficient =

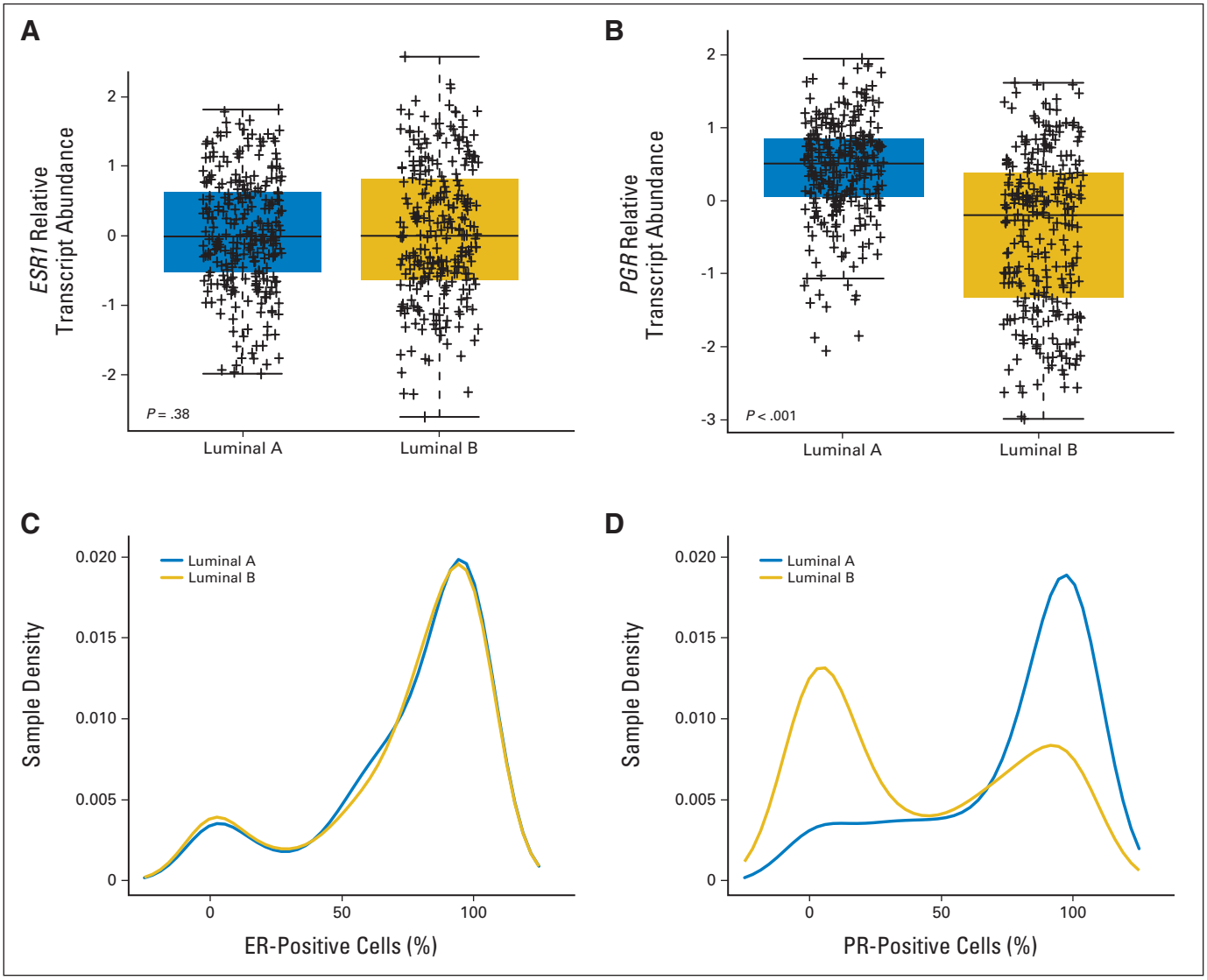

Fig 1. Expression of the hormonal receptors in the Grupo Español de Investigación en Cáncer de Mama 9906 data set. (A) Estrogen receptor (ER) gene (*ESR1*) and (B) progesterone receptor (PR) gene (*PGR*) as assayed using quantitative reverse-transcriptase polymerase chain reaction expression in luminal A and B tumors. Density plots based on the percentage of (C) ER-positive and (D) PR-positive tumor cells as assessed by immunohistochemistry.

−0.19) with the expression of the Ki-67 gene *MKI67*, indicating that these two genes may provide different biologic information.

The mRNA expression-based data suggested that semiquantitative scoring of the PR protein, but not ER protein, might help discriminate the genomically defined luminal A from B tumors. To further explore this hypothesis, we compared the percentage of PR-positive and ER-positive tumor cells as assessed by IHC, among luminal A and B tumors in the GEICAM 9906 cohort,[11] and observed that only the percentage of PR-positive cells can discriminate luminal A from B tumors (Figs 1C and 1D). However, it is important to note that considerable overlap was observed. Finally, PR protein expression was also weakly anticorrelated with Ki-67 protein expression ($r = -0.20$).

Clinicopathologic Features of Luminal A and B Tumors

To identify clinicopathologic differences among the genomically defined luminal A and B tumors, we evaluated the clinicopathologic features of 2,257 patients with luminal A or B primary breast cancer. Across three independent cohorts (Table 1), luminal A tumors showed significantly higher rates of PR positivity, HER2 negativity, histologic grade 1, and tumor stage T0-T1 compared with luminal B tumors. No significant differences in ER status were observed, with the vast majority (92% to 96%) of luminal A and B tumors being ER positive.

IHC-Based Versus PAM50 Subtype Definitions

Current IHC-based definitions of luminal A and B subtypes are imperfect when compared with multigene expression-based assays.[5] To further illustrate this, we evaluated the distribution of the IHC-based definitions within luminal A and B tumors in the BCCA-tamoxifen[6] and the GEICAM 9906 cohorts.[11] As expected, whereas a large majority (81% to 85%) of luminal A tumors were identified as IHC-luminal A, 35% to 52% of luminal B tumors were also identified as IHC-luminal A (Table 2).

Table 1. Clinicopathologic Characteristics of Luminal A and B Tumors

Variable	BCCA-Tamoxifen ER-Positive Only					GEICAM 9906 Node Positive					Combined Microarray Dataset All				
	Luminal A		Luminal B			Luminal A		Luminal B			Luminal A		Luminal B		
	No.	%	No.	%	*P*	No.	%	No.	%	*P*	No.	%	No.	%	*P*
No.	372		329		—	278		264		—	594		414		—
Mean age, years	66.6		67.4		> .05	50.8		51.7		> .05	53.5		55.2		.03
Grade															
1	25	7	5	2	< .001	69	25	26	10	< .001	173	82	38	18	< .001
2	186	54	129	41		141	51	112	42		272	64	152	36	
3	135	39	179	57		68	10	126	47		96	35	176	65	
Nodal positivity	245	72.1	215	69	> .05	—		—		—	220	38	195	49	.002
Tumor size > 2.0 cm	150	44	165	56	.003	136	49	166	63	.0031	341	57	280	68	.001
IHC ER-positive status	—		—		—	257	93	240	92	> .05	552	94	390	95	.583
IHC PR-positive status	248	72	174	56	< .001	261	94	195	74	< .001	206	80	99	66	.001
Clinical HER2-positive status	15	4	30	9	.0067	4	2	37	14	< .001	14	6	19	14	.008

Abbreviations: BCCA, British Columbia Cancer Agency; ER, estrogen receptor; GEICAM, Grupo Español de Investigación en Cáncer de Mama; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; PR, progesterone receptor.

Finally, we explored the survival of the luminal A and B subtypes within the IHC-based luminal A and IHC-luminal B/HER2-negative tumors in the BCCA-tamoxifen cohort[6] (Appendix Table A3, online only). In both cases, luminal A tumors showed a significantly better DRFS outcome than non–luminal A tumors. In multivariable Cox model survival analyses adjusted for histologic grade, age at diagnosis, nodal positivity, and tumor size, the hazard ratio for DRFS in PAM50 luminal A tumors compared with PAM50 non–luminal A was 0.642 within IHC-luminal A tumors (95% CI, 0.422 to 0.975, $P = .038$) and 0.582 within IHC-luminal B/HER2-negative tumors (95% CI, 0.323 to 1.047, $P = .071$).

Survival Outcomes Based on the Percentage of PR-Positive Cells

These data suggested that (1) further improvements in the IHC-luminal A definition is needed because many PAM50-defined luminal B tumors are erroneously identified as IHC-luminal A and (2) quantitative scoring of PR-positive tumor cells, but not ER-positive tumor cells, might help identify good-outcome breast cancers. To test this hypothesis, we evaluated the association of the visually determined percentage of PR-positive and ER-positive invasive breast carcinoma cells with survival outcomes within IHC-luminal A tumors of the BCCA-tamoxifen cohort.[6] As expected, the percentage of PR-positive cancer cells, but not the percentage of ER-positive cancer cells (data not shown), was associated with DRFS after adjusting for standard clinicopathologic variables, with the optimal PR percentage cutoff to predict outcome being found to be 20% (Appendix Fig A2-A3, online only). In contrast, within IHC-luminal B/HER2-negative tumors (ie, HR-positive/Ki-67 > 14%), semiquantitative expression of either PR or ER was not found to be associated with outcome differences (data not shown).

We then tested the prognostic value of the PR cutoff of more than 20% within IHC-luminal A tumors in two independent cohorts of patients with primary breast cancer (GEICAM 9906[11] and the BCCA-no AST cohorts[9]). In both data sets, patients with IHC-luminal A tumors having low positive PR-positive tumor cells (≤ 20%) showed significantly poorer survival compared with tumors with more than 20% of PR-positive tumor cells (Figs 2A and 2B). Multivariable analyses confirmed the independent association between PR expression and survival (Appendix Table A4-A5, online

Table 2. Distribution of the IHC-Based Subtypes Across the Luminal A and B Intrinsic Subtypes

Cohort	IHC-Based Subtypes									
	IHC-Luminal A	%	IHC-Luminal B/ HER2 Negative	%	IHC-Luminal B/ HER2 Positive	%	HER2 Positive	%	Triple Negative	%
BCCA-tamoxifen										
Luminal A	286	81.5	50	14.2	15	4.3	—	—	—	—
Luminal B	109	35.4	169	54.9	30	9.7	—	—	—	—
GEICAM 9906										
Luminal A	231	85.2	32	11.8	4	1.5	0	0	4	1.5
Luminal B	134	51.9	77	29.8	30	11.6	7	2.7	10	3.9

NOTE. Within hormone receptor–positive/HER2-negative disease, the concordance κ value score between the PAM50 luminal A and B definition with the IHC-luminal A and IHC-luminal B/HER2-negative definitions was 0.196 and 0.407 (slight to fair agreement) in the GEICAM 9906 cohorts[11] and BCCA-tamoxifen,[6] respectively.

Abbreviations: BCCA, British Columbia Cancer Agency; GEICAM, Grupo Español de Investigación en Cáncer de Mama; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

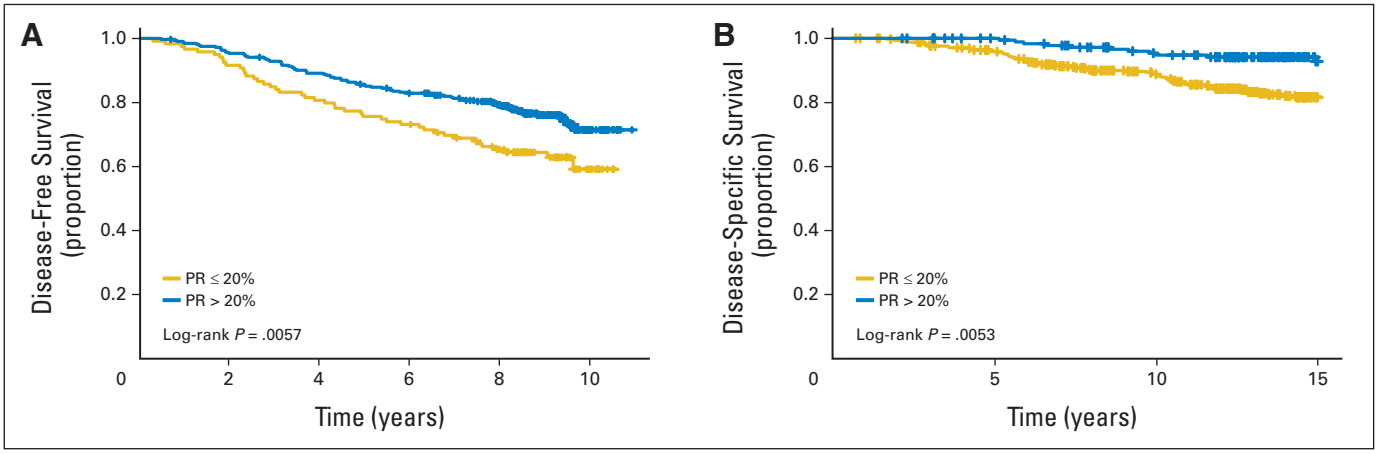

Fig 2. Kaplan-Meier survival analysis within immunohistochemical-based luminal A tumors (hormone receptor positive/HER2 negative/Ki-67 < 14%) based on the percentage of progesterone receptor (PR) –positive tumor cells. (A) Grupo Español de Investigación en Cáncer de Mama 9906 cohort. (B) British Columbia Cancer Agency–no adjuvant systemic therapy cohort.

only). In the BCCA-no AST cohort, the breast cancer–specific survival at 15 years of patients with IHC-luminal A tumors with more than 20% PR-positive tumor cells was 94.0% (95% CI, 91.6% to 98.2%).

We next evaluated the distribution of the gene-expression based intrinsic subtypes (gold standard) within IHC-luminal A tumors in the GEICAM 9906 cohort based on this more than 20% PR cutoff (Table 3). Consistent with the preceding findings, 63% of IHC-luminal A tumors with more than 20% of PR-positive cells were identified as luminal A, whereas 24% of IHC-luminal A tumors with ≤ 20% of PR-positive cells were identified as luminal A, thus confirming that this definition helps to better discriminate true luminal A tumors from the rest. Finally, although the PR cutoff of 20% increased the percentage of luminal A tumors identified within what would otherwise have been considered IHC-luminal B/HER2-negative tumors from 5.9% to 30.9%, the majority of this group remained composed of luminal B (55.6%) tumors.

Comparison of Prognostic Values of IHC-Based Subtypes, IHC4 Score, and PAM50-ROR-P Score

We compared the contribution of the newly proposed IHC-based subtype definitions (IHC-luminal A [HR positive/HER2 negative/Ki-67 < 14%/PR > 20%] and IHC-luminal B [HR positive/HER2 negative/Ki-67 < 14%/PR ≤ 20% or HR positive/HER2 negative/Ki-67 > 14%]) with a version of the IHC4 score[8] and with PAM50 ROR-P score[6] in the subset of patients with HR-positive/HER2-negative tumors from the GEICAM 9906 cohort[11] (n = 580). All three classifications added significant prognostic information beyond clinical variables (Figs 3A, 3B, and 3C), with IHC-based subtypes and IHC4 score providing similar amounts of prognostic information and PAM50 ROR-P providing the largest amount.

Finally, we evaluated the independent prognostic information that each classification provided when considered in the presence of one of the others. When the IHC4 score was included in the model, adding intrinsic IHC-based subtype did not provide significant independent information (Fig 3D). However, when the IHC-based subtype was included in the model, the IHC4 score did not provide additional information (Fig 3E). On the other hand, inclusion of PAM50 ROR-P provided significant independent prognostic information beyond the information provided by either the IHC4 score or the IHC-based subtypes (Figs 3D and 3E).

DISCUSSION

Patients with early breast cancer with tumors that are ER positive and/or PR positive (ie, luminal) have lower risks of recurrence and

Table 3. Distribution of the PAM50 Subtypes Across the Luminal A and B IHC-Based Subtypes in GEICAM 9906

	PAM50 Subtypes										
Subtype and PR Status	Luminal A	%	Luminal B	%	HER2 Enriched	%	Basal-Like	%	Normal-Like	%	Total
IHC-luminal A	231	52.3	134	30.3	56	12.7	3	0.7	18	4.1	442
PR ≤ 20%	27	22.7	61	51.3	24	20.2	3	2.5	4	3.4	119
PR > 20%	204	63.2	73	22.6	32	9.9	0	0	14	4.3	323
IHC-luminal B/HER2 negative	28	21.2	77	58.3	19	14.4	8	6.1	0	0.0	132
PR ≤ 20%	3	5.9	32	62.7	10	19.6	6	11.8	0	0.0	51
PR > 20%	25	30.9	45	55.6	9	11.1	2	2.5	0	0.0	81
IHC-luminal B/HER2 positive	4	5.6	30	41.7	38	52.8	0	0.0	0	0.0	72
PR ≤ 20%	0	0.0	13	33.3	26	66.7	0	0.0	0	0.0	39
PR > 20%	4	12.1	17	51.5	12	36.4	0	0.0	0	0.0	33

Abbreviations: GEICAM, Grupo Español de Investigación en Cáncer de Mama; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; PR, progesterone receptor.

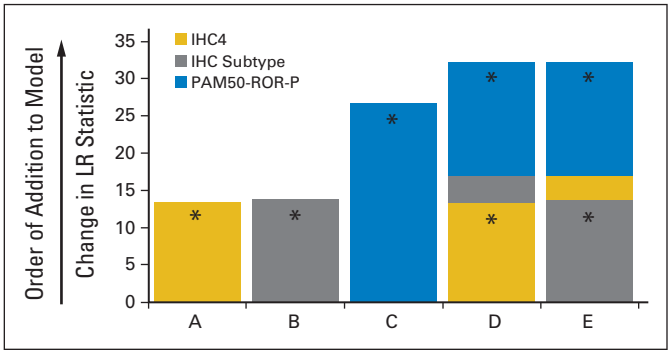

Fig 3. Disease-free survival log likelihood ratio (LR) statistics of six different predictive models (A–E) in patients of the Grupo Español de Investigación en Cáncer de Mama 9906 cohort with hormone receptor (HR) –positive/human epidermal growth factor receptor 2 (HER2) –negative breast cancer. The variables evaluated were the following: immunohistochemical (IHC)-based scoring of estrogen receptor, progesterone receptor, HER2, and Ki-67 (IHC4 score; continuous variable), IHC-based subtypes (HR positive/HER2 negative/Ki-67 $< 14\%$ $> 20\%$ [luminal A], HR positive/HER2 negative/Ki-67 $< 14\% \leq 20\%$ and HR positive/HER2 negative/Ki-67 $> 14\%$ [luminal B]), and PAM50 risk of recurrence score based on subtype and proliferation (ROR-P; continuous variable). (*) $P < .05$.

mortality compared with women with ER-negative and/or PR-negative disease.[3,15] However, few studies have evaluated variations in these risks across ER/PR status.[15-18] In Dunnwald et al,[16] women with ER-positive/PR-negative, ER-negative/PR-positive, or ER-negative/PR-negative tumors experienced higher risks of mortality compared with women with ER-positive/PR-positive tumors, independent of the various demographic and clinical tumor characteristics. These data are concordant with our centrally reviewed pathology data presented here, which show that PR positivity, and especially high expression of PR protein, is more frequently observed in tumors with a better baseline prognosis (ie, luminal A) than tumors with a poor baseline prognosis (ie, luminal B). It is important to note that a substantial number of luminal B tumors (~50% to 75%) are still PR positive, although the expression of PR may be less than in luminal A tumors.

The ability of ER and/or PR expression to predict benefit to endocrine and/or cytotoxic therapy has also been evaluated. In terms of endocrine sensitivity, a recent patient-level meta-analysis of randomized trials from the Early Breast Cancer Trialists' Collaborative Group that evaluated adjuvant tamoxifen versus no adjuvant tamoxifen suggested that recurrence and death rate ratio is independent of PR status (or level) in ER-positive disease.[19] Similar data have been observed in another smaller randomized adjuvant study.[20] In addition, PR expression levels have not shown to predict aromatase inhibitor efficacy over tamoxifen in ER-expressing tumors in two large adjuvant clinical trials.[21,22] This is concordant with a recent neoadjuvant trial in which luminal A and B tumors, as defined by the PAM50 assay, did not show significant differences in terms of response to aromatase inhibitors, although luminal A tumors achieved higher rates of Preoperative Endocrine Prognostic Index score of 0, which is a validated biomarker of outstanding outcome after adjuvant endocrine therapy alone.[23] Overall, these data suggest that luminal A and B tumors benefit similarly from endocrine therapies, but that patients with luminal A tumors have a better baseline prognosis than those with luminal B tumors.

In terms of chemotherapy benefit, the majority of adjuvant and neoadjuvant data suggest that HR status is a strong predictor of general chemosensitivity, with HR-positive tumors showing less benefit to cytotoxic drugs than HR-negative tumors. Moreover, in the neoadjuvant setting, luminal A tumors achieve lower rates of pathologic complete response with anthracycline/taxane-based chemotherapy compared with luminal B tumors.[24] In addition, Oncotype DX has shown that within HR-positive disease, those tumors with high RS (ie, non–luminal A tumors) benefit the most from adjuvant chemotherapy.[25,26] Interestingly, in a retrospective analysis from three adjuvant clinical trials, low expression of both ER and PR, and potentially low expression of PR within ER-positive patients, was found predictive of adding chemotherapy to endocrine therapy.[27] Overall, these data suggest that luminal A tumors are less chemosensitive than luminal Bs.

A critical issue in HR-positive disease is the identification of patients who can be considered virtually cured with endocrine therapy alone and so do not need adjuvant systemic chemotherapy.[4,6] Gene expression–based assays such as the PAM50 ROR and Oncotype DX RS can help identify these groups of patients, especially within node-negative disease.[28] Recently, a combined semiquantitative IHC-based scoring of ER, PR, HER2, and Ki-67, known as IHC4 score, has shown to provide similar prognostic information as is provided by Oncotype DX RS.[8] In this report, we have shown that a version of the IHC4 score is significantly associated with outcome, but did not add significant prognostic information once our newly improved intrinsic IHC-based subtypes were known within HR-positive/HER2-negative disease. This is probably due to the fact that both pathology-based determinations are using the same four biomarkers to identify similar prognostic groups.

There are several issues that need to be considered in this study. First, the information provided by IHC-based biomarkers cannot simply be used to substitute the information coming from multigene-based assays, and even in the presence of IHC-based assays, the gene expression ROR assay was a strong prognostic feature. However, as stated previously, multigene expression-based assays are not globally available, and in their absence, well-designed IHC assays are valuable for baseline prognostic estimations. A second issue is that many genes were found differentially expressed when luminal A tumors were compared with luminal B tumors, and the quantitative IHC expression of some of these biomarkers could have potentially performed better than PR. However, we decided to focus on the expression of PR because this biomarker is widely used in the community and is already part of the standard assessment at most institutions. Third, the IHC-based subtype definitions evaluated here were performed in a centralized laboratory under a single protocol, and one antibody per protein/target, which may not reflect the everyday performance of these tests in the clinical setting, where multiple laboratories with different antibodies is more likely to be the approach. Fourth, the IHC4 score evaluated in our study is slightly different from that of Cuzik et al[8] as a result of the use of different antibodies for ER and PR and the use of a general intensity score of ER-positive tumor cells. Nonetheless, the association of the IHC4 score with survival was found to be strong, as previously reported.[8]

To conclude, IHC subtype–based definitions of genomically defined luminal A and B tumors are imperfect because of the nature and limitations of pathology-based tests. However, semiquantitative measurement of the percentage of PR-positive cells within HR-positive/HER2-negative/Ki-67 less than 14% tumors helps to identify patients who may be considered most effectively treated with endocrine therapy alone. Therefore, the new proposed IHC-based definition of

luminal A tumors is HR-positive/HER2-negative/Ki-67 less than 14% and PR more than 20%.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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