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Research Article

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Intrinsic Subtypes from the PAM50 Gene Expression Assay in a Population-Based Breast Cancer Survivor Cohort: Prognostication of Short- and Long-term Outcomes

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Abstract

Background: The PAM50, a gene expression assay to categorize breast tumors into intrinsic subtypes, has not been previously used to examine short- and long-term prognostication in a population-based cohort where treatment patterns and time of initial follow-up vary.

Methods: In a stratified case–cohort design of 1,691 women from the LACE and Pathways breast cancer survivor cohorts, we used PAM50 to categorize tumors into Luminal A (LumA), Luminal B (LumB), HER2-enriched (HER2-E), Basal-like and Normal-like, and to examine risk of early and late recurrence and mortality by Cox proportional hazards regression.

Results: Compared with LumA, cumulative risk of recurrence and breast cancer death was higher for LumB, HER2-E, and Basal-like tumors at 2, 5, and 10 years. However, HR of breast cancer death varied over time [<5 years (early) vs. > 5 years (late)] for both Basal-like (HR, 6.23 early vs. HR, 0.63 late) and HER2-E tumors (HR, 2.97 early vs. HR, 0.73 late) but not for LumB tumors where risk was elevated consistently (HR, 2.67 early vs. HR, 1.47 late). The contrast between LumB, HER2-E, and Basal-like compared with LumA on early recurrence was stronger when subtype was defined by PAM50 than by immunohistochemistry (IHC) markers.

Conclusions: The PAM50 categorized intrinsic subtypes in a manner that more accurately predicts recurrence and survival, especially for luminal tumors, compared with commonly used methods that rely on traditional IHC clinical markers.

Impact: The PAM50 is robust for use in epidemiologic studies and should be considered when archived tumor tissues are available. *Cancer Epidemiol Biomarkers Prev; 23(5); 725–34. ©2014 AACR.*

Introduction

Breast cancer is a heterogeneous disease with respect to molecular alterations, cellular composition, and clinical outcomes (1–4), and this heterogeneity should be considered in the search for risk factors leading to initiation and progression. Gene expression profiling has given us insight into the molecular complexity of breast tumors (3) and improves prognostication. As a result, many different gene expression tests have been developed. For example, the 21-gene OncotypeDx assay (Genome Health Inc.) can be used to risk stratify early-stage estrogen receptor (ER) positive breast cancer (5, 6), and the 70-gene MammaPrint (Agendia) microarray assay has shown prognostic significance in ER-positive and ER-negative, early-stage, node-negative breast cancer (7, 8). Although these tests seem to accurately predict prognosis or response to chemotherapy, they are applicable only to clinically defined subgroups of breast cancers. There is still controversy over the value that such assays add to clinicopathologic characteristics and the practicing clinician to make informed treatment decisions (9).

To date, however, most research applying gene expression-based assays has been from clinical trial study populations and not breast cancers treated in the community or prospective epidemiologic cohorts. Beyond their potential utility for individual care, molecular subtyping may be useful to incorporate in epidemiologic research. Subtyping may enable us to understand underlying factors specific to biologic pathways and how behavioral and lifestyle risk factors differ by molecular subgroup.

One of the more recently developed genomic assays, the PAM50, is based on the intrinsic subtypes that have become commonly known as Luminal A (LumA), Luminal B (LumB), HER2-enriched (HER2-E), Basal-like, and Normal-like (3, 10, 11) and can be applied across all clinical subgroups of breast cancer. The test can be performed on formalin-fixed, paraffin-embedded (FFPE)

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tissues, and thus is particularly useful for epidemiologic studies where fresh tissue is typically not available. Previous studies with the PAM50 have been done to assess "pure" prognosis (i.e., in patients who received no systemic therapy; ref. 3), and in randomized clinical trials to assess subtype response to different chemotherapy regimens (12, 13). Furthermore, follow-up time in these studies have been relatively short and did not examine prognostication in the latter years postdiagnosis.

Our goal was to evaluate the performance of the PAM50 in a population-based study in a combined group of breast cancer survivors from 2 cohort studies where treatment patterns and time of initial follow-up varied among patients. The parent cohorts were diverse in terms of race and ethnicity, and included a broad range of ages at diagnosis and disease severity. In addition, we were interested in the performance of the PAM50 in predicting early versus late outcomes, and how the performance of the PAM50 in this population differed from subtype classifications utilizing immunohistochemical (IHC) and pathologic markers routinely collected in community settings.

Materials and Methods

Study population

Women were patients with breast cancer from 2 population-based cohorts, the LACE, and Pathways cohorts. LACE participants were 18 to 79 years old at breast cancer diagnosis, diagnosed with early stage breast cancer from 1996 to 2000 (AJCC stage I with tumor size $\geq$1 cm, stage II or stage IIIA) and at the time of entry into the cohort had completed chemotherapy or radiation therapy, and were within 39 months of diagnosis (median time from diagnosis to enrollment = 23 months, 61% between 12 and 24 months). At the time of enrollment into the LACE cohort, women were required to be free of recurrence. The majority of women were identified from the Kaiser Permanente Northern California (KPNC, 83%) and the University of Utah Cancer Registries (12%). LACE study methods and baseline characteristics of participants have been described (14). The Pathways study enrolled women diagnosed with invasive breast cancer from 2005 to 2013 in KPNC with no previous diagnosis of other invasive cancer, and were at least 21 years of age at diagnosis; most women were enrolled within 2 months of diagnosis (mean time from diagnosis to enrollment = 1.8 months, maximum 7.2 months). Women were identified from daily review of pathology reports. Details of the study methods have been previously described (15). For this study, we included Pathways women diagnosed through 2008. Additional exclusions for this study were invasive tumor <0.5-cm diameter, bilateral disease, or neoadjuvant therapy. Participants provided informed consent under protocols approved by institutional review boards at KPNC and the University of Utah.

Patient and disease characteristics at the time of diagnosis, including age, disease stage, tumor size, node status, and histologic grade, were abstracted from tumor registry data and medical records review. Ethnicity was based on self-report. Hormone receptor status (ER and PR) and Her2 expression were obtained from medical record review and either the KPNC Cancer Registry (KPNC cases) or Utah Cancer Registry (Utah cases). For all breast surgical specimens at KPNC, ER, PR, and Her2 status were determined by immunohistochemistry (IHC) at the KPNC regional IHC lab; at Utah, by hospital pathology departments or ARUP Laboratories, Inc. (Salt Lake City, UT).

A total of 1,691 women were selected for PAM50 molecular subtyping. We used a stratified case–cohort study design, with strata based on IHC results of ER, PR, and Her2. The case–cohort design is an efficient alternative to the nested case–control study design in studies examining multiple outcomes (e.g., recurrence and survival; ref. 16). This design consists of a random sample of a subcohort from the parent cohort with follow-up for all outcomes of interest. In addition, all non-subcohort members of the parent cohort with any outcome of interest during follow-up are selected into the study. Rather than simple random sampling of the subcohort, we selected a stratified random sample given the potential for increasing statistical efficiency in analyses. ER, PR, and Her2 status based on IHC (and/or FISH for HER2) defined the strata used for sampling, with an 18% random sample selected among cases of the common breast cancer phenotype that is positive for ER or PR expression and negative for Her2 (and has low risk of recurrence) and a 100% sample of tumors that were ER^- and PR^- or $Her2^+$. Table 1 describes details of the sample selection.

Table 1. Details of the sample selection

Clinical subtype	Population, *N*	Sampled, *N*	Sample fraction	Extra cases, *N*	Subcohort after exclusions	Extra cases after exclusions	Analytic sample
ER^+ or PR^+, $Her2^-$	3,018	500	18%	467	435	372	
ER^+ or PR^+, $Her2^+$	439	439	100%		343		
ER^-, PR^-, $Her2^-$	505	505	100%		405		
ER^-, PR^-, $Her2^+$	177	177	100%		136		
Totals	4,139	1,621		467	1,319	372	1,691

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After exclusions, there were 372 non-subcohort members with an event of interest (recurrence, second breast cancer, death) that were included in the study, all of whom were from the nonsampled remaining women with ER^+ or PR^+, $Her2^-$ tumors. The cohort was followed for recurrence and survival through August, 2012. Among all events, 370 women had a recurrence and 510 died of any cause, with 274 (53.7%) from breast cancer.

Outcomes were ascertained by self-report and regular linkage to medical records and KPNC mortality files and verified by medical record review. Cause of death was determined from death certificates and supplemented with medical records if necessary. Primary analytic outcomes were: new breast cancer event, defined as a first recurrence/metastasis or new primary breast cancer (hereafter referred to as recurrence) and breast cancer–specific mortality.

Tissue samples

For cohort members who were selected for the analytic sample, we contacted the hospital where surgery for resection of the primary tumor was performed, or the institution's pathology storage facility, to obtain FFPE tissue blocks from the procedure and corresponding slides. Slides were reviewed by one pathologist (R.E. Factor). The pathologist marked an area of representative tumor tissue on a slide.

From the 2,088 women selected for the case–cohort (1,621 subcohort and 467 extra cases), 150 (7.2%) had no suitable tumor block available. For an additional 67 (3.2%), we were unable to obtain consent to retrieve the tumor block and in 24 women (1.2%), the PAM50 assay failed. An additional 155 women were ineligible if the area of invasive tumor was observed to be smaller than 0.5 cm in diameter, if the appearance of the primary tumor tissue in the slides indicated to the pathologist that neoadjuvant therapy had been used before resection, or if the pathology report indicated bilateral disease. For eligible cases, tissue punches 1 mm in diameter were obtained from the area of the FFPE tissue block corresponding to the marked slide. Two punches per case (or one punch if the primary tumor was less than 0.7 cm in diameter) were placed in plastic tubes labeled with a sample identifying number.

Gene expression assay and IHC categorization

Tissue punches were deparaffinized and digested for RNA extraction as described previously (17). Reverse transcriptase PCR (RT-PCR) was conducted for the 50 target genes (i.e., PAM50) and 5 control genes (3). Details of RT-PCR methods have been provided elsewhere (17, 18). Laboratory personnel were blinded to clinical information and received only a study identifying number to track the sample. Each batch of tissue samples sent to the laboratory for assay work included a mix of clinicopathologic types.

Surrogate subtyping was done using available clinical IHC results for ER and PR, and clinical IHC and/or FISH for Her2. Scoring criteria for each clinical marker followed the standard at the time of diagnosis. Subtyping by IHC using the 3 standard markers was the foundation for the sampling and weighting in the cohort (Table 2). Recent recommendations for clinicopathologic categorization of breast tumor subtypes incorporate these 3 markers with the addition of Ki-67 proliferation score or histologic tumor grade (19) and PR status (20) to distinguish low- and high-risk endocrine positive tumors. Accordingly, for data analysis, we used 2 different methods of subtype classification, that were both modifications of more commonly used methods. The first classification was an adaptation of the Carey method (21), herein called the 3-marker IHC method adapted from Carey, which categorized tumors that were ER^+ or PR^+ and $Her2^-$ as "LumA," ER^+ or PR^+ and $Her2^+$ as "LumB," ER^-, PR^-, and $Her2^+$ as "$Her2^+/ER^-$," and ER^- and PR^- and $Her2^-$ as triple negative (TNBC). The original Carey method incorporated CK5/6 and HER1, which were not available to us. The second classification incorporated grade in place of Ki-67 and defined subtype according to categories adopted by the St. Gallen's Consensus Conference (19) with a further adaptation by Prat (20). We herein referred to this as the 3-marker IHC plus grade adapted from St. Gallen's and is defined as follows: low-risk endocrine positive or surrogate "LumA" as ER^+ and PR^+ and $Her2^-$ (well-differentiated or moderately differentiated); high-risk endocrine positive or surrogate "LumB" as ER^+ or PR^+ and any of PR^-, $Her2^+$, or tumor grade of poorly or undifferentiated; "Her2-positive, endocrine negative" as ER^-, PR^-, and $Her2^+$; and TNBC as ER^-, PR^-, and $Her2^-$.

To determine molecular subtypes from the PAM50 data, we used centroids from an independent RT-qPCR training set (17). For each sample, this algorithm generates a categorical subtype call, a Pearson correlation to each subtype in the training set, and a continuous quantitative score (between 1 and 10) for the expression of *ESR1*, *PGR*, *ERBB2*, and proliferation.

Data analysis

All analyses incorporated sampling weights and the stratified sampling design for unbiased estimation of population parameters and valid estimates of standard errors using the "svy" commands in Stata software, StataCorp. This method includes estimates of frequency distributions of baseline characteristics. The Cox proportional hazards regression model was used to estimate HR and 95% confidence intervals (CI) for the associations of PAM50 subtype with recurrence and breast cancer–specific mortality, adjusted for age at diagnosis, race/ethnicity, tumor size, number of positive nodes and grade. Time since diagnosis was the time scale used in the regression models, allowing for delayed entry into the cohort (i.e., left truncation, with time of entry into the study ranging from 0 to 3.2 years postdiagnosis). Point and interval estimation of regression parameters accounted for the case–cohort study design with stratified sampling of the subcohort using the methods of Borgan and Langholz and

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Table 2. Characteristics of the stratified case–cohort study population

	LACE ($n = 872$) Weighted %	Pathways ($n = 766$) Weighted %	Total ($n = 1,638$) Weighted %	*P* value
Age at breast cancer diagnosis				
<50	22.2	22.9	22.5	0.82
50–64	45.2	46.5	45.9	
65+	32.6	30.7	31.5	
Race/ethnicity				
White	81.6	66.3	73.3	<0.0001
Black/AA	5.5	6.7	6.2	
Hispanic	6.2	10.8	8.7	
Asian/PI	5.5	12.8	9.5	
Other	1.2	3.4	2.4	
Education				
Higher secondary or less	26.1	16.4	20.8	<0.0001
Some college	39.6	39.4	39.5	
College grad	15.2	25.2	20.6	
Post-college	19.0	19.0	19.0	
Family history				
No	75.7	80.3	78.2	0.12
Yes	24.3	19.7	21.8	
Smoking history at breast cancer diagnosis				
Never	51.9	52.7	52.3	0.92
Past	41.7	41.5	41.6	
Current	6.4	5.8	6.0	
Menopausal status at breast cancer diagnosis				
Postmenopausal	66.5	71.8	69.4	<0.0001
Premenopausal	19.4	28.2	24.2	
Unknown	14.0	0.0	6.4	
BMI				
Under/normal	46.8	35.3	40.5	0.001
Overweight	28.8	30.7	29.8	
Obese	24.5	34.1	29.7	
AJCC stage				
I	45.7	51.6	48.9	<0.0001
II	51.5	37.0	43.6	
III	2.8	10.4	6.9	
IV	0.0	1.0	0.5	
Tumor size				
<2 cm	62.3	65.2	63.9	0.001
≥2 cm	37.7	31.6	34.4	
Missing	0.0	3.2	1.7	
Number of positive nodes				
0	65.2	64.1	64.6	0.001
1–4	27.0	24.6	25.7	
5+	7.8	7.5	7.6	
Missing	0.0	3.8	2.1	
Tumor grade				
I	19.3	23.5	21.6	0.11
II	40.1	43.5	42.0	
III–IV	31.5	26.8	28.9	
Missing	9.1	6.1	7.5	

(Continued on the following page)

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Table 2. Characteristics of the stratified case–cohort study population (Cont'd)

	LACE ($n = 872$) Weighted %	Pathways ($n = 766$) Weighted %	Total ($n = 1,638$) Weighted %	*P* value
Breast cancer surgery				
None	0.0	1.3	0.7	<0.0001
Lumpectomy	50.1	62.5	56.8	
Mastectomy	49.9	36.3	42.5	
Chemotherapy				
No	46.5	52.6	49.8	0.08
Yes	53.5	47.4	50.2	
Radiation				
No	38.7	66.0	53.5	<0.0001
Yes	61.3	34.0	46.5	
Hormonal therapy				
No	22.6	27.3	25.2	0.06
Yes	77.4	72.7	74.8	
Any comorbidity (Charlson comorbidity index)				
No	87.0	85.2	86.0	0.45
Yes	13.0	14.8	14.0	
ER status				
Negative	18.4	16.8	17.5	0.36
Positive	81.6	83.2	82.5	
PR status				
Negative	35.4	31.6	33.3	0.20
Positive	64.6	68.4	66.7	
Her2 status				
Negative	83.4	87.0	85.3	0.02
Positive	16.6	13.0	14.7	

colleagues, as implemented in SAS subroutines developed by Langholz and Jia (22).

We further conducted analyses stratified by PAM50 subtype and IHC categorization as defined by an adaptation of the Carey method (21) and an adaption by Prat (20) of the recommendation from the St. Gallen's Consensus Conference (17). In addition, we examined heterogeneity in strength of association between PAM50 subtype and risk over time (<5 years, 5–10 years) via introduction of cross-product terms between time and subtype.

Results

We obtained PAM50 assay results from the tumors of 1,691 women within a combined cohort of 4,139 breast cancer survivors participating in the LACE and Pathways studies. The 53 tumors classified as normal-like by PAM50 were excluded from the analysis, for a final analytical sample size of 1,638. During a mean follow-up of 7.4 years, we identified 370 recurrences and 274 breast cancer deaths. A total of 243 recurrences and 115 breast cancer deaths occurred in the first 5 years after diagnosis.

Table 2 provides distributions of selected characteristic for the LACE and Pathway cohorts, based on applying the sampling weights for the case–cohort patients included in this analysis. LACE and Pathways women were similar in age, family history of breast cancer, smoking history, and receipt of chemotherapy. Pathways enrolled a higher percentage of women who were minority (33.7% vs. 18.4%), college-educated (83.6% vs. 73%), and who were obese [body mass index (BMI) > 30; 34.1% vs. 24.5%]. These distributions are very similar to what has been reported on the full cohort (ref. 23; Table 2). With respect to tumor characteristics, Pathways had a higher percentage of women with stage III tumors (10.4% vs. 2.8%), and had a lower percentage of women who were $Her2^{+}$ (54% vs. 44.0%). Treatment also varied between Pathways and LACE women: LACE women, diagnosed in 1996 to 2000, more frequently had a mastectomy (49.9% vs. 36.3%) and more frequently had radiation therapy (61.3% vs. 34.0%) than the more recently diagnosed (2005–2008) Pathways women.

Breast cancer mortality in the cohort differed markedly by PAM50 subtype. Among women with LumA tumors, the cumulative probability of dying from breast cancer increased from <1% at 2 years to 7.1% at 10 years but remained considerably lower than all other subtypes at every time point examined. Risk of dying from breast cancer was highest for Basal-like tumors, most markedly at 2 and 5 years. LumB and HER2-E tumors were similar at 2 and 5 years, but LumB had a worse cumulative mortality after 10 years. At 10 years, cumulative risk of breast cancer

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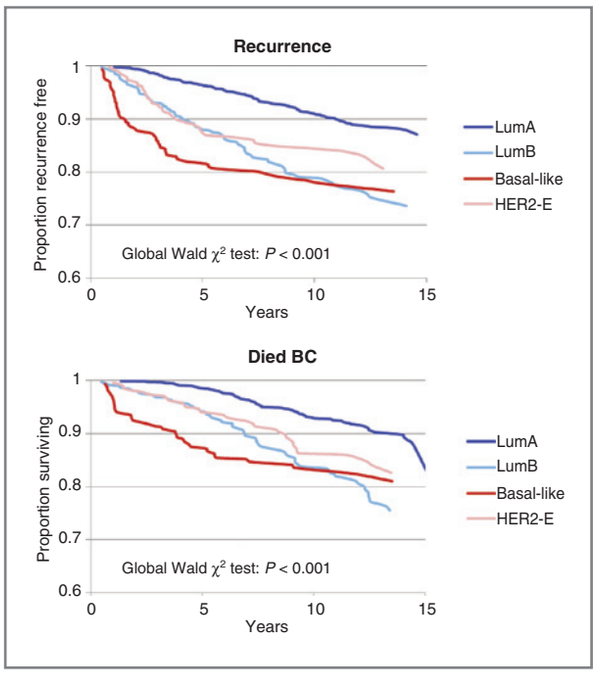

Figure 1. Kaplan–Meier estimates for breast cancer survival and recurrence, by PAM50 subtype.

death was highest for those with Basal-like (17.0%) and LumB (16.2%) subtypes (Fig. 1).

We also examined the difference in survival from Basal-like tumors by time of recruitment into the cohort (range: 0.3–3.1 years) to determine the impact of delayed follow-up commonly observed in epidemiologic cohorts. Figure 2 demonstrates the difference in cumulative hazard for Basal-like and LumB tumors in Pathways women who entered the cohort close to diagnosis (average 2 months postdiagnosis) versus LACE participants who entered the cohort after completion of surgery and chemotherapy (average 22 months postdiagnosis). For the LACE women who needed to survive this 22-month period on average and be recurrence-free at the time of study follow-up, the subsequent risk of dying of breast cancer among women with Basal-like tumors seemed similar to risk of women with LumA tumors, whereas Pathways women who only needed to survive a couple of months and not be free of recurrence and had basal-like tumors had a significantly elevated mortality risk compared with those with LumA. For LumB tumors, risk was slightly higher in Pathways than in LACE compared with LumA but risk from the LACE and Pathways cohorts seemed to converge at approximately 5 years.

Table 3 shows the examination of PAM50 subtypes on risk of recurrence adjusted for age, race/ethnicity, tumor size, number of positive nodes, and grade. Women who were classified by PAM50 as having LumB (HR, 1.94; 95% CI, 1.36–2.77), HER2-E (HR, 1.63; 95% CI, 1.10–2.41), or Basal-like (HR, 2.10; 95% CI, 1.37–3.22) subtypes all had a significantly greater risk of recurrence compared with those who were classified as LumA when both early and late recurrence events were considered together. Women who were HER2-E and treated with Herceptin

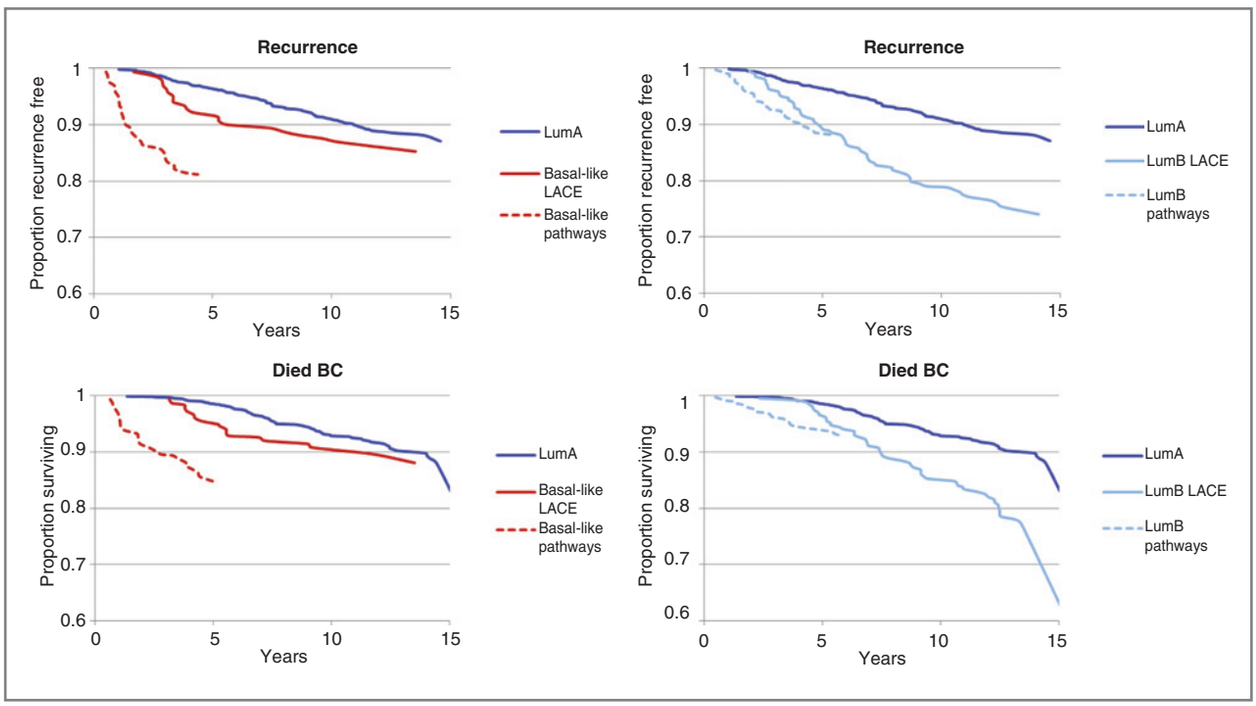

Figure 2. Kaplan–Meier estimates for breast cancer survival and recurrence, by early entry (Pathways study) vs. late entry (LACE study) for Basal-like and LumB tumors compared with LumA tumors.

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Table 3. Risk of recurrence by PAM50 intrinsic subtype

	All recurrences, $n = 370$				Early recurrences (<5 years), $n = 243$				Late recurrences (5–10 years), $n = 127$			
PAM50 subtype	*N* events	HR	Lower 95% CI	Upper 95% CI	*N* events	HR	Lower 95% CI	Upper 95% CI	*N* events	HR	Lower 95% CI	Upper 95% CI
LumA	127		Ref		61		Ref		66		Ref	
LumB	120	1.94	1.36	2.77	82	2.55	1.68	3.88	38	1.34	0.79	2.28
LumB Pathways	53	1.54	0.99	2.41	52	2.39	1.48	3.86	1	0.14	0.02	1.08
LumB LACE	67	2.42	1.54	3.82	30	2.93	1.67	5.14	37	1.73	0.99	3.04
HER2-E	67	1.63	1.10	2.41	54	2.83	1.83	4.38	13	0.55	0.28	1.09
Basal-like	56	2.10	1.37	3.22	46	3.98	2.47	6.42	10	0.59	0.28	1.23
Basal-like Pathways	33	3.83	2.33	6.28	33	5.44	3.23	9.15	0			
Basal-like LACE	23	1.29	0.75	2.23	13	2.41	1.23	4.75	10	0.63	0.30	1.33

NOTE: Adjusted for age, race/ethnicity, tumor size, number of positive nodes, and grade.

had fewer recurrences and better survival than those not treated with Herceptin when both were compared with LumA (data not shown). When the risks for only early recurrences (relapse in the first 5 years after diagnosis) were considered, HRs for each subtype compared with LumA were of considerably higher magnitude than HRs for events occurring at all timepoints combined. The risk of early recurrence for Basal-like (HR, 3.98; 95% CI, 2.47–6.42) was almost twice as high as for all timepoints combined, and for HER2-E (HR, 2.83; 95% CI, 1.83–4.38) approximately 75% higher. The risk for LumB for early events (HR, 2.55; 95% CI, 1.68–3.88) was similar to risk for all timepoints, indicating that risk remained relatively constant over time.

Table 4 shows the risk of breast cancer death by PAM50 subtype adjusted for race/ethnicity, tumor size, number of positive nodes and grade. Risk of breast cancer death for women with Basal-like tumors varied most by time. For events occurring in the first 5 years, the risk of breast cancer death is more than 6 times higher than that of LumA (HR, 6.23; 95% CI, 3.31–11.73). However, once a woman survives to 5 years without an event, those with Basal-like tumors were no longer at increased risk for breast cancer death (HR, 0.61; 95% CI, 0.30–1.23) compared with LumA subtype. The risk for HER2-E subtype compared with LumA also varied by time of breast cancer death, but differences in HRs for risk of breast cancer death (HR, 2.97 early vs. HR, 0.73 late) were not as pronounced as for Basal-like tumors. Differences by time are least pronounced in LumB tumors (HR, 2.67 early vs. HR, 1.47 late); risk of breast cancer death was only slightly lower in events occurring >5 years compared with events <5 years.

To exemplify how results may differ depending on methods used to classify tumors into subtypes, we used the risk of early recurrence as the outcome (see Table 5) to present differences in risk of early recurrence by 2 subtype classification methods defined by IHC and IHC and grade compared with PAM50. For each classification method, the non LumA subtype was compared with the LumA subtype, which served as the reference group.

Table 4. Risk of breast cancer death by PAM50 intrinsic subtype

	All breast cancer deaths, $n = 274$				Early breast cancer deaths (< 5 years), $n = 115$				Late breast cancer deaths (5–10 years), $n = 154$			
PAM50 subtype	*N* events	HR	Lower 95% CI	Upper 95% CI	*N* events	HR	Lower 95% CI	Upper 95% CI	*N* events	HR	Lower 95% CI	Upper 95% CI
LumA	96		Ref		23		Ref		73		Ref	
LumB	86	1.77	1.16	2.70	35	2.67	1.46	4.88	49	1.47	0.87	2.48
LumB-Pathways	29	1.42	0.81	2.47	26	3.01	1.55	5.85	3	0.43	0.12	1.49
LumB-LACE	57	2.03	1.23	3.35	9	2.03	0.86	4.81	48	1.74	1.01	3.01
HER2-E	46	1.27	0.79	2.02	24	2.97	1.58	5.59	22	0.73	0.40	1.33
Basal-like	46	1.84	1.12	3.01	33	6.23	3.31	11.73	13	0.61	0.30	1.23
Basal-like Pathways	25	4.74	2.64	8.51	24	8.60	4.38	16.88	1	0.86	0.11	6.61
Basal-like LACE	21	1.05	0.58	1.93	9	3.63	1.54	8.56	12	0.59	0.29	1.22

NOTE: Adjusted for age, race/ethnicity, tumor size, number of positive nodes, and grade.

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Table 5. Early recurrences: comparison of PAM50 and IHC tumor subtype classifications

	Early recurrences ($n = 243$)			
	***N* events**	**HR**	**Lower 95% CI**	**Upper 95% CI**
PAM50 subtype[a]				
LumA	61		Ref	
LumB	82	2.55	1.68	3.88
HER2-E	54	2.83	1.83	4.38
Basal	46	3.98	2.47	6.42
Carey subtype defined by IHC[a]				
"LumA" surrogate	142		Ref	
"LumB" surrogate	31	1.19	0.79	1.81
$Her2^{+}/ER^{-}$	16	1.37	0.78	2.38
TNBC	54	2.13	1.46	3.11
Adapted by Prat from St. Gallens Consensus conference: subtype defined by IHC[b]				
"LumA" surrogate	69		ref	
"LumB" surrogate	104	1.77	1.22	2.56
$Her2^{+}/ER^{-}$	16	2.05	1.16	3.62
TNBC	54	3.25	2.22	4.77

[a]Adjusted for age, race/ethnicity, tumor size, number of positive nodes, and grade.
[b]Adjusted for age, race/ethnicity, tumor size, and number of positive nodes.

Risk associated for LumB subtype (PAM50) or IHC proxy for LumB varied by method used. When defined by PAM50 (HR, 2.55; 95% CI, 1.68–3.88), risk of early recurrence was statistically significant compared with LumA and was approximately double the risk defined by the 3-marker IHC adaptation of the Carey method (HR, 1.19; 95% CI, 0.79–1.81; 21), but estimates were closer to, but still higher than, the 3-marker IHC plus grade method adapted from the St. Gallen Consensus Conference (HR, 1.77; 95% CI, 1.22–2.56; ref. 20). For both HER2-E and Basal-like tumor subtypes, risk of an early recurrence was again higher when defined by PAM50 than when defined by the 3-marker IHC adaptation of the Carey method and closer to, but still higher than, the 3-marker IHC plus grade adaptation of the St. Gallen method. Triple negatives defined by the 3-marker IHC adaptation of the Carey method had slightly more than half the risk of early recurrence (HR, 2.13; 95% CI, 1.46–3.11) than PAM50 Basal-like tumors (HR, 4.07; 95% CI, 2.53–6.56).

Discussion

This study is innovative in using PAM50, a gene expression assay, rather than IHC, to investigate the prognostication of intrinsic subtypes in a population-based epidemiologic cohort of breast cancer survivors. The cohort represents a heterogeneous group in which cases vary by ER, PR, and Her2 status, pathologic characteristics, adjuvant therapy, and initiation and length of follow-up. We demonstrated that when using PAM50 to characterize tumors, women with LumA tumors had significantly lower risk of recurrence and breast cancer death than women with more aggressive tumor subtypes (LumB, HER2-E, and Basal-like) and higher risks for poor outcomes. We also demonstrated that the PAM50 when compared with the routinely collected clinical IHC markers (ER, PR, and Her2) and tumor grade to categorize women, seemed better able to distinguish risk groups within luminal subtypes, specifically those with the probability of the lower risk of recurrence and breast cancer death (LumA) from those with higher risk (LumB). This is consistent with improved risk prediction observed for PAM50 in clinical study populations (12, 24).

In a study of 786 women who were ER-positive by IHC, Nielsen and colleagues demonstrated that when subtyped subsequently by PAM50, 9% were reassigned to non-Luminal subtypes (24) and women reassigned to either LumB, or the non-Luminal subtypes (HER2-E and Basal-like), had significantly worse prognosis than women who remained in the LumA category. Similarly, in another study of 476 node positive premenopausal women diagnosed between 1989 and 1993 and randomized to receive either adjuvant CEF or CMF (12), patients with HER2-E, Basal-like, and LumB subtypes by PAM50 all had significantly higher risk of a poor clinical outcome compared with women with LumA tumors, regardless of treatment arm.

Our study was able to demonstrate that PAM50 was most useful in risk prediction for the early period postdiagnosis (0–5 years) and less useful in the later period postdiagnosis (5–10 years). We demonstrated elevated risk of all subtypes compared with LumA in the first 5 years, with the Basal-like tumors conferring the highest magnitude of risk and higher than other subtypes. In addition, after 5 years, risks for HER2-E and Basal-like

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subtypes compared with LumA were no longer increased, and only risk for LumB remained significantly increased.

Using estimates from Adjuvant! Online to determine disease-specific survival (similar to our risk of death from breast cancer), Nielson and colleagues (24) reported risks stratified by early risk (0–5 years postdiagnosis) and late risk (5–10 years postdiagnosis) by intrinsic subtype. They found increased risks of similar magnitude to our findings. Women with HER2-E (RR 3.65) and LumB (RR 1.99) had increased risks of disease-specific survival compared with LumA, and Basal-like tumors had the largest increased risk (RR, 17.71), which was similar to our findings (RR, 8.60, for those entering the cohort closer to diagnosis). They also reported that the risk of LumB compared with LumA remained significantly elevated in the 5- to 10-year period and only slightly decreased from risk estimates of 0 to 5 years, while the significantly increased risk observed for HER2-E in the early postdiagnosis period was no longer increased in the 5- to 10-year period postdiagnosis. This is also consistent with data from another study from Bianchini and colleagues (25) of more than 1,500 ER-positive women, which demonstrated that those tumors with high proliferation as well as high estrogen gene expression were those who were at highest risk for late relapses after 5 years (HR, 3.86; $P = 0.007$) when compared with those with high proliferation and low estrogen expression.

Other studies have also found that subtypes from PAM50 seem better able to predict those with poorer outcomes compared with using IHC markers (ER, PR, and Her2) and tumor grade to categorize women (3, 17). In a study by Parker and colleagues (3) where they examined the risk of relapse models using PAM50 and compared them to models using pathologic stage, grade, and routine IHC biomarker status (ER and Her2), there was clear improvement in prediction with subtype relative to the model employing clinical variables only. Furthermore, a combination of clinical variables and subtype was also a significant improvement over either individual predictor variables or subtype alone. However, information on grade did not significantly improve risk of relapse in the combined model, indicating that the prognostic value of grade had been superseded by information provided by the intrinsic subtype.

Lastly of relevance in epidemiologic studies where all follow-up may not start at diagnosis and the PAM50 is used, we found that the distribution of subtypes may be biased toward less aggressive tumor subtypes because women with tumors that recurred early or died from breast cancer are likely excluded from the study sample. This "survivor bias" impacts the prognostic value of the PAM50 because tumors that remain in the more aggressive subtypes of HER2-E and Basal-like are likely to be composed of the least aggressive tumors within that subtype.

In conclusion, the PAM50 had excellent prognostic value in a population-based sample where treatment, demographic, and clinicopathologic characteristics are not uniform, where a portion of the most aggressive tumors resulting in early recurrence/death were likely to be excluded because of delayed follow-up and where treatment with Herceptin varied because some women were diagnosed before it became available. Our results suggest the utility of this assay is robust for defining molecular tumor subtypes in this situation. Although a recent task force (9) on use of molecular subtypes for clinical practice concluded that there is not adequate evidence to use PAM50 subtypes to make treatment decisions, the PAM50 seems well suited for incorporation into population-based epidemiologic studies of breast cancer survivorship. In particular, using the PAM50 should be considered in such studies when archived (FFPE) tumor samples are available to help examine if risk factors for breast cancer survival vary by tumor subtype. For epidemiologic studies, the test seems to categorize intrinsic subtypes in a manner that more accurately predicts survival compared with commonly used methods that rely on traditional IHC clinical markers.

Disclosure of Potential Conflicts of Interest

L.A. Habel has a commercial research grant from bioTheranostics. P.S. Bernard has ownership interest (including patents) in University Genomics and Bioclassifier. No potential conflicts of interest were disclosed by the other authors.

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