

## TIM-3 expression in breast cancer

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### ABSTRACT

Tumor-infiltrating lymphocytes (TILs) are predominantly present in breast cancer patients with estrogen receptor negative tumors, among whom increasing levels correlate with favorable outcomes. Nevertheless, currently available immune checkpoint inhibitors appear to benefit only a small number of women with breast cancer. Upregulation of additional immune checkpoint markers is one mechanism of resistance to current inhibitors that might be amenable to targeting with newer agents. T-cell Immunoglobulin and Mucin domain-containing molecule 3 (TIM-3) is an immune checkpoint receptor that is an emerging target for cancer immunotherapy. We investigated TIM-3 immunohistochemical expression in 3,992 breast cancer specimens assembled into tissue microarrays, linked to detailed outcome, clinico-pathological parameters and biomarkers including CD8, PD-1, PD-L1 and LAG-3. We scored and reported absolute counts for TIM-3+ intra-epithelial and stromal TILs (iTILs and sTILs), and find that breast cancer patients with TIM-3+ iTILs ( $\geq 1$ ) represent a minority of cases (11%), with a predilection for basal-like breast cancers (among which 28% had TIM-3+ iTILs). TIM-3+ sTILs ( $\geq 2$ ) represented 20% of cases and included more non-basal cases. The presence of TIM-3+ iTILs highly correlates with hematoxylin and eosin-stained stromal TILs and with other immune checkpoint markers (PD-1+ iTILs, LAG-3+ iTILs and PD-L1+ tumors). In prognostic analyses, early breast cancer patients with TIM-3+ iTILs have significantly improved breast cancer-specific survival whereas TIM-3+ sTILs did not reach statistical significance. In multivariate analyses, the presence of TIM-3+ iTILs is an independent favorable prognostic factor in the whole cohort as well as among ER negative patients. Our study supports TIM-3 as a target for breast cancer immunotherapy.

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## Introduction

The presence of small round dark mononuclear cells characteristic of tumor-infiltrating lymphocytes (TILs) on hematoxylin and eosin (H&E) – stained breast cancer specimens has garnered increased attention with the emergence of immune checkpoint inhibitors and has led to a re-examination of the role of the immune system in breast tumors. Accumulating evidence shows that the presence of an immune response in breast cancers correlates with estrogen receptor negative (ER-) subtypes (i.e. the HER2 and basal-like intrinsic subtypes) among whom there is an association with favorable outcomes<sup>1–3</sup>. In contrast, the more common ER+ breast cancer subtypes rarely display such heightened immune responses, which when present are associated with unfavorable prognosis<sup>4–6</sup>.

Immune checkpoint inhibitors targeting cytotoxic T-Lymphocyte-associated antigen 4 (CTLA-4), programmed cell death-1 (PD-1) or its ligand (PDL-1) perform best in immunogenic cancers such as melanoma and non-small cell lung cancer<sup>7,8</sup>, but responses have recently been reported in triple negative/basal-like breast cancers<sup>9–11</sup> (for reviews see refs. <sup>12,13</sup>). However, even among such potentially immunogenic cancers, immune checkpoint inhibitors benefit only a

relatively small number of patients<sup>7,12,14–18</sup>. As resistance may be due to the activation of alternative checkpoint pathways, additional immune checkpoints targets have become a subject of active research, including the T-cell Immunoglobulin and Mucin-domain-containing molecule 3 (TIM-3)<sup>19</sup>.

TIM-3 is an immune receptor discovered in 2002 that is expressed on a variety of immune cells including dendritic cells, macrophages, and T cells<sup>20–22</sup>. TIM-3 mediates its suppressive activity on immune cells via its ligands that include phosphatidylserine, CEACAM-1 and the widely expressed ligand galectin-9<sup>23,24</sup>. TIM-3 is expressed on activated T cells and its signaling on cytotoxic T cells leads to an exhausted phenotype, characterized by a reduction in proliferation, decreased production of effector cytokines and apoptosis of effector T cells<sup>25</sup>. In addition, TIM-3+ TILs can co-express PD-1, with blockade of both receptors leading to a more pronounced tumor regression than either agent alone, at least in pre-clinical studies<sup>26,27</sup>.

Multiple studies have now reported on the presence of TIM-3+ TILs in human tumors<sup>28–32</sup>. However, in breast cancer, TIM-3+ TILs have been evaluated by immunohistochemistry in a limited number of patients, with one recent study reporting positive associations with lymph node metastases<sup>33,34</sup>. The objective of our study is to evaluate the expression of TIM-3 on

TILs in a large series of breast cancers powered for multivariate correlation with clinico-pathological parameters, survival, and other important immune biomarkers.

## Results

### Distribution of TIM-3+ iTILs in breast cancers

To define staining conditions and interpretation, we conducted an initial evaluation of TIM-3 staining and correlation with clinico-pathological parameters on a TMA consisting of 330 breast cancer patients (representing a training set). We observed 12% of breast cancer cases with TIM-3+ intratumoral tumor infiltrating lymphocytes ( $\geq 1$  iTIL per 0.6 mm diameter core) whereas stromal TIM-3+ sTILs ( $\geq 1$ ) were present in 48% of cases (Supplemental Table 1). We then proceeded with TIM-3 staining on a TMA comprising an independent cohort of 3,992 breast cancer cases, of which 3,148 cases were interpretable for TIM-3 immunohistochemistry staining (Supplemental Figure 1). The results were consistent with the training set as 11% of cases had  $\geq 1$  TIM-3+ iTILs and 40% of cases had  $\geq 1$  TIM-3+ sTILs (Supplemental Figure 2). TIM-3 expression on macrophages was only observed in 1% of cases and was not analyzed further.

As there were a large number of cases with TIM-3+ sTILs, a cut-off for dichotomization of  $\geq 2$  sTIL/0.6 mm core, a level reached in 20% of breast cancers, was selected based on analyses of Kaplan Meier curves of different TIM-3+ sTILs cutpoints (as described in Methods: *Statistics*). However, TIM-3+ iTILs were selected as the primary analysis parameter, to allow comparison with previously published immune biomarkers in this breast cancer cohort<sup>35,36</sup>.

**Table 1.** TIM-3+ iTILs association with clinico-pathological parameters in breast cancer.

Parameters	Negative N = 2816	TIM-3+ iTILs $\geq 1$ N = 332 (11%)	P-value* ( $\chi^2$ )
<b>Age at diagnosis (years)</b>			
< 50	787	119 (13%)	0.003
$\geq 50$	2029	213 (10%)	
<b>Tumor size (cm)</b>			
$\leq 2$	1467	163 (10%)	0.331
$> 2$	1334	166 (11%)	
<b>Grade</b>			
1&2	1286	96 (7%)	< 0.001
3	1414	219 (13%)	
Unknown	116	12	
<b>Ki67</b>			
Negative (< 14%)	1469	100 (6%)	< 0.001
Positive ( $\geq 14%$ )	1085	209 (16%)	
Unknown	262	23	
<b>ER</b>			
Negative	716	159 (18%)	< 0.001
Positive (> 1%)	2091	172 (8%)	
Unknown	9	1	
<b>Subtypes</b>			
Luminal A	1209	73 (6%)	< 0.001
Luminal B	631	80 (11%)	
HER2E	184	25 (12%)	
Basal-like	205	81 (28%)	
Triple negative, non-basal	182	40 (18%)	
Unknown	240	17	

\* Chi-square test

### The presence of TIM-3+ iTILs in breast cancer is associated with unfavorable clinico-pathological factors

Consistent with the results in the initial cohort of 330 patients, breast cancer cases with TIM-3+ iTILs in the validation cohort were significantly associated with younger age at presentation, higher grade, hormone receptor (ER/PR) negativity, and high Ki67 proliferation index [defined as  $\geq 14%$ ] (Table 1). In addition, the presence of TIM-3+ iTILs was much more common in the basal-like subtype relative to other subtypes (28% in basal-like vs 6% in luminal A). The results for TIM-3+ sTILs reflected similar associations with clinico-pathological parameters to TIM-3+ iTILs findings (Supplemental Table 2). However, more of the non-basal cases had TIM-3+ sTILs than was the case for iTILs.

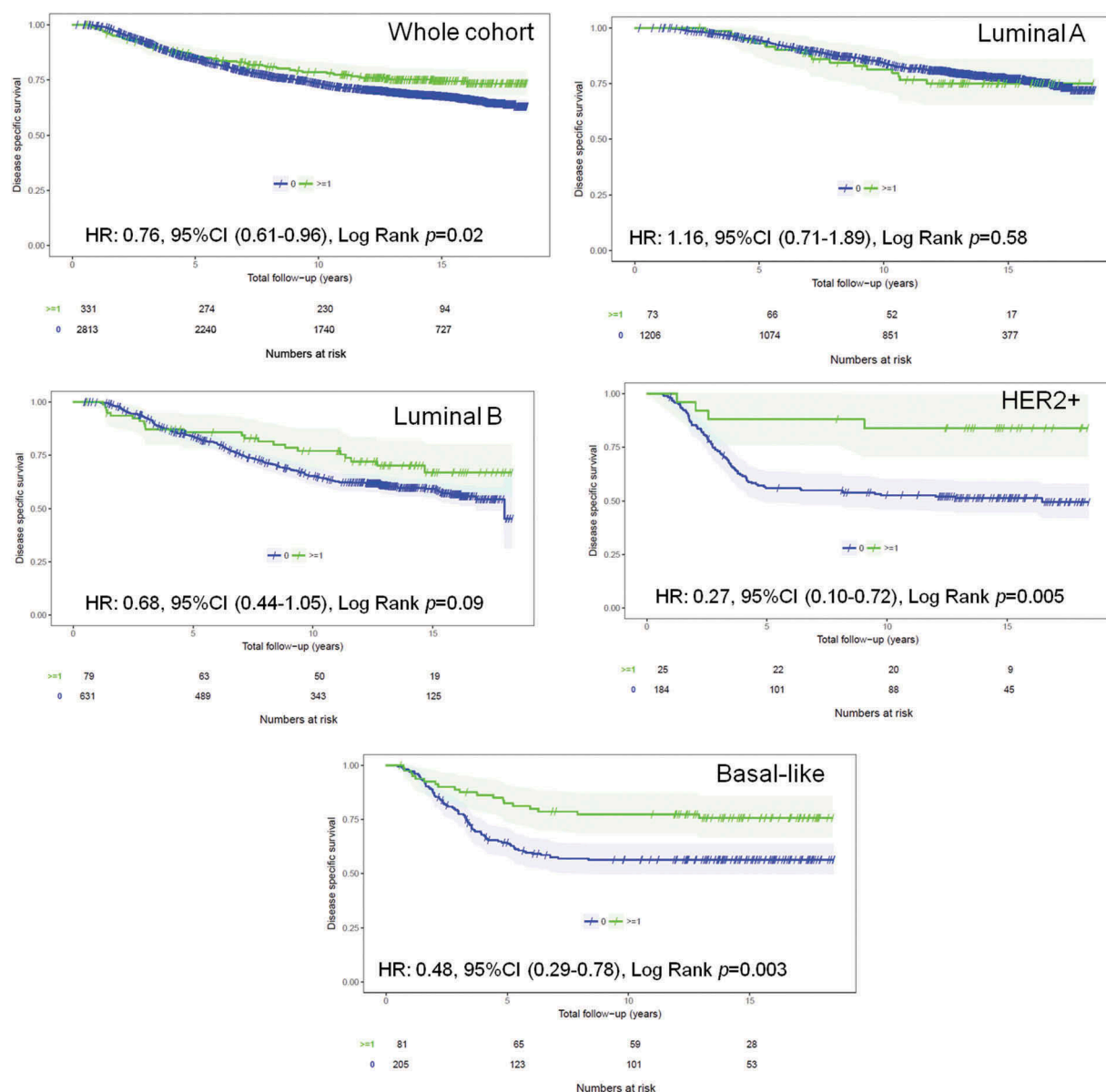
### TIM-3+ iTILs correlate with the presence of other immune checkpoint markers (LAG-3, PD-1, PD-L1) and overall H&E stils

Because this large cohort had been previously assessed for key immune biomarkers including PD-1, PD-L1 and LAG-3, we were able to analyze their correlations with TIM-3. In addition, we scored overall H&E stromal TILs to allow a parallel evaluation with immune checkpoint markers.

We found that breast tumors with TIM-3+ iTILs were highly significantly associated with the presence of additional immune checkpoint markers (Table 2). Indeed, nearly half of breast cancers that are positive for PD-L1 or PD-1+ iTILs or LAG-3+ iTILs were also infiltrated with TIM-3+ iTILs in the same 0.6 mm TMA core. However, only 3% (91/2736 interpretable cases) expressed all three immune checkpoint markers (TIM-3+/PD-1+/LAG-3+) (Table 2) when assessed by this method. We did not observe any particularly unique association pattern between the presence of TIM-3+ iTILs and any of the other individual immune checkpoint markers tested, suggesting that the TIM-3 checkpoint expression on TILs occur in tumors containing T cells positive for other exhausted markers. Furthermore, we found that all immune checkpoint markers correlated positively ( $p < 0.001$ ) with H&E sTILs (Supplemental Figure 3). In this cohort, less than 1% of cases were categorized as lymphocyte-predominant breast cancer (LPBC, defined as  $\geq 50%$  H&E sTILs).

### TIM-3+ iTILs are associated with good prognosis in early breast cancer

In univariate analyses, the presence of TIM-3+ iTILs in early breast tumors was associated with improved breast cancer-specific survival (BCSS) in the whole cohort (HR: 0.76, 95%CI 0.61–0.96, Log Rank  $p = 0.02$ ) (Figure 1). When breast cancer subtypes were stratified in the analysis, only HER2+ and basal-like breast cancer patients with TIM-3+ iTILs displayed significantly improved BCSS (**HER2+**: HR: 0.27, 95%CI 0.10–0.72, Log Rank  $p = 0.005$ ; **Basal-like**: HR: 0.48, 95%CI 0.29–0.78, Log Rank  $p = 0.003$ ) (Figure 1). These results were similar using overall survival and relapse-free survival secondary endpoints (Supplemental Figure 4 for overall survival; Supplemental Figure 5 for relapse-free survival). In contrast,



**Figure 1. TIM-3+ iTILs association with BCSS in the whole (validation) cohort and by breast cancer subtype.** Kaplan Meier curves of breast cancer-specific survival in breast cancer patients stratified by the presence or absence of TIM-3+ iTILs. KM curves in (A) the whole cohort, (B) Luminal A cases, (C) Luminal B, (D) HER2+ and (E) basal-like cases are shown with their corresponding numbers of patients, events and log rank  $p$  values. The number of patients still at risk at the end of each 5 years of follow-up is shown at the bottom of each panel.

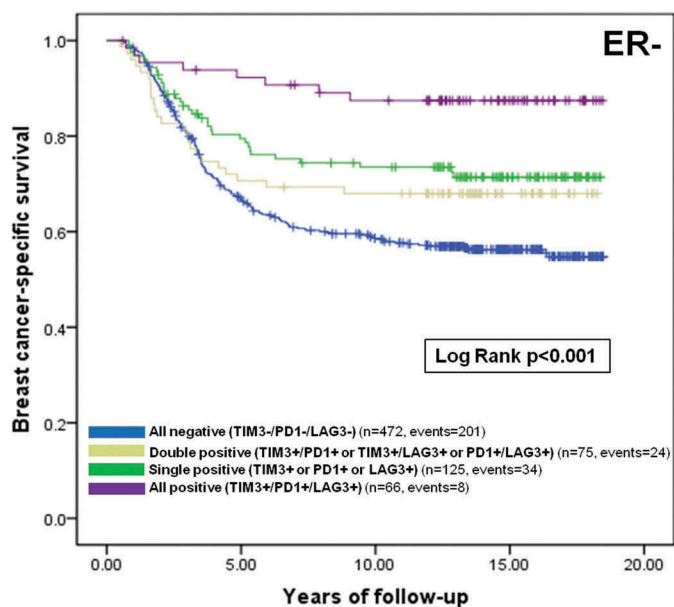
in exploratory analyses, the presence of TIM-3+ sTILs had a trend for favorable prognosis for BCSS and relapse-free survival and reached significance in the whole cohort for overall survival (Supplemental Figure 6 for BCSS; Supplemental Figure 7 for overall survival and Supplemental Figure 8 for relapse-free survival).

In multivariate analyses that included H&E sTILs as a covariate, the presence of TIM-3+ iTILs remained a favorable prognostic factor in the whole cohort and ER- breast cancer patients (Table 3 – **Whole cohort**: HR: 0.64, 95%CI 0.48–0.85,  $p = 0.001$ ; **ER-**: HR: 0.58, 95%CI 0.39–0.86,  $p = 0.004$ , **Basal-like**: HR: 0.58, 95%CI 0.32–1.03,  $p = 0.052$ ). Similar findings were observed for TIM-3+ sTILs albeit not reaching significance for basal-like breast cancer patients (Supplemental Table 3). We also found that ER- breast cancer patients with tumors that were co-infiltrated with TIM-3+, PD-1+ and

LAG-3+ TILs had a significant improved breast cancer specific survival, in univariate and multivariate analyses, relative to patients with a single positive, dual positive, or complete absence of these three immune checkpoint markers (Figure 2, Table 4).

## Discussion

We report the first study of TIM-3 expression in a large (> 1000 case) series of early breast cancers. TIM-3 expression in this cohort was restricted to tumor-infiltrating lymphocytes and was present in about 12% of cases when 0.6 mm cores were evaluated for expression on intra-epithelial TILs, or 20% of cases when assessed on stromal TILs. The presence of TIM-3+ iTILs was associated with younger age, high grade and high Ki67 proliferation index and was enriched in the basal-



**Figure 2. Prognostic value of TIM-3, PD-1 and LAG-3+ iTILs co-infiltration among ER- breast cancer patients.** Kaplan Meier curve of breast cancer-specific survival among ER- breast cancer patients stratified by the presence or absence of one or more immune checkpoint markers is shown with corresponding number of patients, events and a log rank p value. *Blue*: All negative (TIM3-/PD1-/LAG3-), *green*: Single positive (TIM3+ or PD1+ or LAG3+), *grey*: Double positive (TIM3+/PD1+ or TIM3+/LAG3+ or PD1+/LAG3+), *purple*: All positive (TIM3+/PD1+/LAG3+).

**Table 2.** Association of TIM-3+ iTILs with other immune biomarkers in breast cancer.

Immune biomarkers	TIM-3+ iTILs = 0 (n = 2816)	TIM-3+ iTILs $\geq$ 1 (n = 332)	P-value ( $\chi^2$ )
PD-L1*			
Negative	2374	215 (8%)	< 0.0001
Positive ( $\geq$ 1%)	133	<b>97 (42%)</b>	
PD-1+ iTILs*			
Negative	2388	198 (8%)	< 0.0001
Positive ( $\geq$ 1)	127	<b>113 (47%)</b>	
LAG-3+ iTILs*			
Negative	2344	169 (7%)	< 0.0001
Positive ( $\geq$ 1)	175	<b>146 (45%)</b>	
CD8+ iTILs*			
Negative	1881	111 (6%)	< 0.0001
Positive ( $\geq$ 1)	778	<b>213 (22%)</b>	

\*Frequency in the whole cohort: **PD-L1  $\geq$  1%** = 241/2918 (8.3%); **PD-1+ iTILs  $\geq$  1** = 246/2908 (8.5%); **LAG-3+ iTILs** = 327/2921 (11%), from Burugu S et al., *Annals of Oncology*, 2017. **CD8+ iTILs  $\geq$  1** = 1089/3403 (32%) from Liu S et al., *Breast Cancer Research*, 2012)

like breast cancer subtype. Moreover, TIM-3+ iTILs highly correlated with co-infiltration of additional immune checkpoint markers PD-L1 (on carcinoma cells), PD-1 and LAG-3+ (on TILs). In prognostic analyses, early breast cancer patients with TIM-3+ iTILs had significantly improved survival for all assessed endpoints, as compared to patients whose tumors lacked TIM-3+ iTILs. In multivariate analyses, the prognostic effect was maintained in the whole cohort as well as among ER- and basal-like breast cancer patients.

Studies from our group and from others have been consistent in finding that the presence of immune checkpoint markers on intra-epithelial TILs in breast tumors is an uncommon event, and mostly restricted to ER- breast cancers<sup>33,36,37</sup>. However, TILs positive for immune checkpoint

markers are able to discriminate breast cancer patients with favorable survival, consistent with an active anticancer immune microenvironment. Indeed, we found that breast tumors infiltrated with TIM-3+ iTILs highly correlate with tumors positive for other checkpoint markers (PD-1, PD-L1 and LAG-3). Results are consistent with other reported studies and imply that the expression of multiple different immune checkpoints can occur during tumor progression, reflecting an ongoing battle between cancer cells and the immune system<sup>38,39</sup>. In our cohort, coexpression of TIM-3 with PD-1 and LAG-3 is associated with a particularly favorable prognosis, perhaps reflecting an underlying robust immune recognition of the cancer cells that is difficult for the tumor to evade. Furthermore, other studies have reported TIM-3 expression on carcinoma cells to be associated with poor prognosis (for meta-analysis see ref. <sup>40</sup>), which we did not observe in our large breast cancer cohort. These apparently conflicting results may be due to the different types of tumor and possible confounding by stage or other factors, as the smaller studies in other tumors were not powered for multivariate analyses.

Strengths of our study include the use of a large cohort of early breast cancer patients, treated consistently according to provincial guidelines, linked to detailed long-term outcome data and assessed using a training and validation approach to biomarker interpretation. Some limitations include, first, the necessity in such a large series to rely on TMA cores, representing a 0.28 mm<sup>2</sup> surface area sampling of a tumor for assessment of the tumor immune microenvironment. Second, infiltration of TILs bearing multiple immune biomarkers could only be inferred from single stains and therefore does not directly identify co-expression on the same lymphocyte. Third, breast cancer patients in the cohort received what would now be considered older treatments (predating trastuzumab, taxanes and aromatase inhibitors) which may affect extrapolation of some of the observed prognostic and predictive associations to more contemporary treatment regimens.

Accumulating evidence suggests resistance to anti-CTLA-4 or anti-PD-1/PD-L1 inhibitors can occur in otherwise immunogenic cancers through compensatory upregulation of additional immune checkpoints<sup>39,41</sup>. TIM-3 has recently emerged as a target for cancer immunotherapy following pre-clinical studies suggesting its non-redundant functions in comparison to the better-characterized checkpoint markers PD-1/PD-L1, and efficacious treatment synergy when TIM-3 is targeted in combination with anti-PD1/PDL1 antibodies<sup>26,27,42,43</sup>. Although ER- breast cancer, in particular basal and triple negative breast cancer, is considered the most immunogenic subtype, reports from immune checkpoint inhibitor clinical trials are not as encouraging. Early reports suggest metastatic breast cancer patients may benefit most from PD-1/PD-L1 blockade monotherapy in the first-line setting, or in combination with chemotherapy agents for second or third-line therapy with an objective response rate ranging from 10%-40%<sup>9-11</sup> (for review, see ref. <sup>12</sup>). The findings from our study imply that TIM-3 inhibitors could potentially help to treat PD-1 refractory or metastatic tumors. Currently, four early phase clinical trials testing the efficacy of anti-TIM-3 in combination with anti-PD-1/PDL1 in advanced tumors have

**Table 3.** Multivariate analyses of TIM-3+ iTILs in the whole cohort, among ER- and in basal-like patients for breast cancer-specific survival including H&E sTILs as a covariate.

Whole cohort (# of events/n: 705/2379)		Hazard Ratio for BCSS (95% CI)	LRT P-value
<b>Age at diagnosis</b> (Reference group: > 50)	≥ 50	1.06 (0.86–1.30)	0.61
<b>Tumor grade</b> (Reference group: grade 1–2)	Grade 3	1.49 (1.26–1.77)	< 0.001
<b>Tumor size</b> (Reference group: ≤ 2cm)	> 2	1.63 (1.39–1.91)	< 0.001
<b>Lymphovascular invasion status</b> (Reference group: negative)	Positive	1.33 (1.11–1.60)	0.002
<b>Nodal status</b> (Reference group: negative)	Positive	2.29 (1.86–2.82)	< 0.001
<b>Adjuvant systemic therapy</b> (Reference group: no AST)	TAM only	0.73 (0.57–0.93)	0.05
	Chemo only	0.74 (0.56–0.99)	
	TAM+ chemo	0.69 (0.49–0.97)	
<b>Breast cancer subtypes</b> (Reference group: Luminal A)	Luminal B/Ki67	1.81 (1.50–2.19)	< 0.001
	Luminal/HER2+	2.16 (1.64–2.84)	
	HER2+	2.54 (1.93–3.35)	
	Basal-like	2.28 (1.74–2.99)	
<b>H&amp;E sTILs</b> (10% increments)		0.98 (0.98–0.99)	< 0.001
<b>TIM-3+ iTILs</b> (Reference group: 0)	≥ 1	0.64 (0.48–0.85)	0.001
Among ER-* (# of events/n: 255/705)		Hazard Ratio for BCSS (95% CI)	LRT P-value
<b>Age at diagnosis</b> (Reference group: > 50)	≥ 50	0.90 (0.66–1.23)	0.50
<b>Tumor grade</b> (Reference group: grade 1–2)	Grade 3	1.91 (1.35–2.70)	< 0.001
<b>Tumor size</b> (Reference group: ≤ 2cm)	> 2	1.62 (1.23–2.12)	0.001
<b>Lymphovascular invasion status</b> (Reference group: negative)	Positive	1.32 (0.99–1.77)	0.06
<b>Nodal status</b> (Reference group: negative)	Positive	2.44 (1.76–3.38)	< 0.001
<b>Adjuvant systemic therapy</b> (Reference group: no AST)	TAM only	0.89 (0.59–1.34)	0.64
	Chemo only	0.81 (0.55–1.19)	
	TAM+ chemo	1.02 (0.59–1.75)	
<b>H&amp;E sTILs</b> (10% increments)		0.98(0.97–0.99)	0.002
<b>TIM-3+ iTILs</b> (Reference group: 0)	≥ 1	0.58 (0.39–0.86)	0.004
Among basal-like (# of events/n: 94/263)		Hazard Ratio for BCSS (95% CI)	LRT P-value
<b>Age at diagnosis</b> (Reference group: < 50)	≥ 50	0.86 (0.50–1.46)	0.57
<b>Tumor grade</b> (Reference group: grade 1–2)	Grade 3	1.39 (0.72–2.71)	0.31
<b>Tumor size</b> (Reference group: ≤ 2cm)	> 2	1.40 (0.91–2.16)	0.13
<b>Lymphovascular invasion status</b> (Reference group: negative)	Positive	1.31 (0.82–2.10)	0.26
<b>Nodal status</b> (Reference group: negative)	Positive	2.01 (1.19–3.38)	0.008
<b>Adjuvant systemic therapy</b> (Reference group: no AST)	TAM only	1.65 (0.80–3.42)	0.60
	Chemo only	1.21 (0.65–2.25)	
	TAM+ chemo	1.40 (0.47–4.19)	
<b>H&amp;E sTILs</b> (10% increments)		0.97 (0.95–0.99)	0.002
<b>TIM-3+ iTILs</b> (Reference group: 0)	≥ 1	0.58 (0.32–1.03)	0.052

\* including HER2 positive and negative

opened [NCT03066648, NCT02608268, NCT02817633, and NCT03099109]. Our data support that this appears to be a relevant combinatorial strategy to assess in breast cancer, particularly in patients with non-BRCA mutated basal-like tumors, an aggressive subtype for which targeted therapies are not currently available.

## Methods

### Study cohorts

A training set consisting of 330 breast cancer patients was used to finalize biomarker staining and interpretation conditions for an initial analysis of TIM-3. These patients were

**Table 4.** Multivariate analyses of TIM-3/PD-1/LAG-3+ iTILs among ER- breast cancer patients for BCSS.

Among ER- (# of events/n: 249/686)		Hazard Ratio for BCSS (95% CI)	P-value*
<b>Age at diagnosis</b> (Reference group: < 50)	≥ 50	0.89 (0.64–1.24)	0.49
<b>Tumor grade</b> (Reference group: grade 1–2)	Grade 3	2.22 (1.56–3.16)	< 0.001
<b>Tumor size</b> (Reference group: ≤ 2cm)	> 2	1.50 (1.14–1.98)	0.004
<b>Lymphovascular invasion status</b> (Reference group: negative)	Positive	1.355 (0.998–1.840)	0.052
<b>Nodal status</b> (Reference group: negative)	Positive	2.654 (1.887–3.731)	< 0.001
<b>Adjuvant systemic therapy</b> (Reference group: no AST)	TAM only	0.851 (0.566–1.279)	0.436
	Chemo only	0.739 (0.495–1.103)	
	TAM+ chemo	1.193 (0.683–2.084)	
<b>TIM-3/PD-1/LAG-3+ iTILs</b> (Reference group: TIM3-/PD1-/LAG3-)	TIM3-/PD1+/LAG3-	0.499 (0.251–0.989)	0.046
	TIM3-/PD1-/LAG3+	0.498 (0.289–0.861)	0.012
	TIM3-/PD1+/LAG3+	0.586 (0.299–1.148)	0.119
	TIM3+/PD1-/LAG3-	0.615 (0.314–1.203)	0.155
	TIM3+/PD1+/LAG3-	0.348 (0.086–1.410)	0.139
	TIM3+/PD1-/LAG3+	0.959 (0.519–1.772)	0.893
	TIM3+/PD1+/LAG3+	0.165 (0.073–0.375)	< 0.001

\*Wald-test

diagnosed with invasive breast cancer at the University of British Columbia hospitals between 1989 and 2002 and have been previously described<sup>44</sup>. A detailed description of the validation set consisting of 3,992 breast cancer patients has been previously published<sup>45,46</sup>. In brief, newly diagnosed invasive breast cancers from centres across the province of British Columbia performing breast cancer excision surgery, referred to the British Columbia Cancer Agency (BCCA) between 1986 and 1992 and for which both blocks from a central estrogen receptor testing laboratory and detailed clinico-pathologic, treatment and outcome data collected by the BCCA Breast Cancer Outcomes Unit were available were assembled into 17 single core tissue microarray blocks. None of these patients (training and validation cohorts) received neoadjuvant treatment. Table 1 summarizes the basic clinico-pathological parameters of the study populations. The median follow-up for both cohorts is 13 years. The Clinical Research Ethics Board of the University of British Columbia and the British Columbia Cancer Agency Breast Cancer Outcomes Unit approved the access to the samples and corresponding de-identified outcome data.

### Immunohistochemistry and scoring

Tissue microarrays (TMAs) were built from formalin-fixed paraffin-embedded primary excision specimens from patients in the training and validation cohorts and represented as 0.6mm cores across 3 blocks for the training cohort and 17 blocks for the validation cohort. These TMAs have been previously stained and scored for multiple biomarkers including ER, PR, HER2, Ki67, EGFR, CK5/6, CD8, LAG-3, PD-1 and PD-L1<sup>36</sup>. Breast cancer intrinsic subtypes were previously determined from both cohorts by immunohistochemistry (IHC) benchmarked against a gene expression gold standard (the PAM50 intrinsic subtype classifier)<sup>47</sup>. Briefly, ER+ (≥ 1%) or PR+ (≥ 1%), HER2- (including IHC 2+ cases that were HER2- by fluorescence in situ hybridization) and low (< 14%) Ki67 were defined as Luminal A; hormone receptor

positive cases which were also either HER2+ or had high Ki67 were defined as Luminal B; HER2+/ER-/PR- cases were defined as HER2E, and triple negative cases that were positive for EGFR+ or CK5/6+ were defined as basal-like.

Overall stromal TILs were scored on H&E-scanned images of the TMA cores using the assessment recommendations of the International TILs Working Group<sup>48</sup>, whereby stromal TILs are scored as the percentage of intertumoral stromal surface area (i.e. excluding areas occupied by carcinoma cells) containing mononuclear lymphocytic infiltrates.

TIM-3 immunohistochemistry was conducted with anti-TIM-3 rabbit monoclonal antibody clone D5D5R from Cell Signaling (Cat# 45208) as employed in other publications<sup>21,33,49,50</sup>, here using a Ventana Ultra automated stainer (Ventana Medical Systems) in concordance with manufacturer's protocol. In brief, slides underwent antigen retrieval with Standard Cell Conditioning 1 reagent (Ventana Medical Systems) followed by 60 minutes of primary antibody incubation (applied at 1:50 dilution) with no heat, and visualized using a chromoMap DAB detection kit (Ventana Medical Systems). Membranous staining in tonsil tissue served as a positive control in each staining run. TIM-3+ lymphocytes scores were reported as absolute counts per TMA core for intra-epithelial or stromal locations. TIM-3+ intra-epithelial lymphocytes (TIM-3+ iTILs) were defined as TIM-3+ lymphocytes located within carcinoma nests whereas TIM-3+ stromal lymphocytes (TIM-3+ sTILs) were those not in direct contact with the carcinoma nest.

### Statistics

IBM SPSS software (version 24.0) and R (version 3.3.2) were used to conduct all the statistical analyses. TIM-3+ iTILs scores were dichotomized ≥ 1 (as positive) vs. 0 (as negative). In addition, TIM-3 expression on other immune cells (non-lymphocytes) was assessed, but as only 1% of cases were positive on the training set this staining pattern was not further analyzed.

For prognostic analyses, the primary end-point, breast cancer-specific survival, was defined as the time from date of diagnosis to date of death attributed to breast cancer. Patients were censored at death from another cause or if alive at end of follow-up. Relapse-free survival and overall survival were secondary end-points. Relapse-free survival was defined as time from date of diagnosis to date of any type of breast cancer relapse (local, regional, distant, or contralateral) and overall survival as time from date of diagnosis to date of death, irrespective of the cause of death. Correlation with survival was conducted using Kaplan-Meier curves, log-rank test and Cox regression models. Proportional hazard assumptions were assessed by visual examinations of Kaplan-Meier plots. The effect size was adjusted in multivariate Cox regression models by taking into account significant clinicopathological parameters (age, tumor grade, tumor size, lymphovascular invasion and nodal status).

Clinico-pathological and prognostic associations for TIM-3 + iTILs were analyzed first on the training cohort (n = 330) and further tested on the validation cohort (n = 3,992) in a pre-specified formal written statistical plan, presented at the British Columbia Cancer Agency Breast Cancer Outcomes Unit. Furthermore, half of the validation cohort served for a training and a validation approach specifically for correlations and combinatorial analyses among immune biomarkers (TIM-3, PD-L1, PD-1, LAG-3, CD8) due to the low number of positive cases observed in the training set. In addition, 40% of cases in the validation cohort were considered TIM-3 + sTIL positive based on a  $\geq 1$  positive TIL cut-point. A cut-point of  $\geq 2$  positive TILs for TIM-3+ sTILs, representing 20% of cases, was selected following testing of various cut-points ( $\geq 1$ ,  $\geq 2$ ) based on the distribution on half of the validation cohort set in prognostic analyses. In these cases, a pre-specified written statistical plan for validation on the other half of the set was presented prior to statistical analyses. Prognostic analyses of co-infiltrated immune checkpoint markers were nevertheless considered exploratory. All statistical tests performed were two-sided at  $\alpha = 0.05$ .

## Disclosure

Torsten O. Nielsen reports a proprietary interest in the Prosigna test for breast cancer intrinsic subtype, not part of the current study. All other co-authors have declared no conflicts of interest.

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