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Combining histological grade, TILs, and the PD-1/PD-L1 pathway to identify immunogenic tumors and de-escalate radiotherapy in early breast cancer: a secondary analysis of a randomized clinical trial

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ABSTRACT

Background The implementation of immunological biomarkers for radiotherapy (RT) individualization in breast cancer requires consideration of tumor-intrinsic factors. This study aimed to investigate whether the integration of histological grade, tumor-infiltrating lymphocytes (TILs), programmed cell death protein-1 (PD-1), and programmed death ligand-1 (PD-L1) can identify tumors with aggressive characteristics that can be downgraded regarding the need for RT.

Methods The SweBCG91RT trial included 1178 patients with stage I–IIA breast cancer, randomized to breast-conserving surgery with or without adjuvant RT, and followed for a median time of 15.2 years. Immunohistochemical analyses of TILs, PD-1, and PD-L1 were performed. An activated immune response was defined as stromal TILs ≥10% and PD-1 and/or PD-L1 expression in ≥1% of lymphocytes. Tumors were categorized as high-risk or low-risk using assessments of histological grade and proliferation as measured by gene expression. The risk of ipsilateral breast tumor recurrence (IBTR) and benefit of RT were then analyzed with 10 years follow-up based on the integration of immune activation and tumor-intrinsic risk group.

Results Among high-risk tumors, an activated immune infiltrate was associated with a reduced risk of IBTR (HR 0.34, 95% CI 0.16 to 0.73, p=0.006). The incidence of IBTR in this group was 12.1% (5.6–25.0) without RT and 4.4% (1.1–16.3) with RT. In contrast, the incidence of IBTR in the high-risk group without an activated immune infiltrate was 29.6% (21.4–40.2) without RT and 12.8% (6.6–23.9) with RT. Among low-risk tumors, no evidence of a favorable prognostic effect of an activated immune infiltrate was seen (HR 2.0, 95% CI 0.87 to 4.6, p=0.100).

Conclusions Integrating histological grade and immunological biomarkers can identify tumors with aggressive characteristics but a low risk of IBTR despite a lack of RT boost and systemic therapy. Among high-risk tumors, the risk reduction of IBTR conferred by an activated immune infiltrate is comparable to treatment with RT. These findings may apply to cohorts dominated by estrogen receptor-positive tumors.

BACKGROUND

Adjuvant radiotherapy (RT) after breast-conserving surgery (BCS) significantly decreases the incidence of ipsilateral breast tumor recurrence (IBTR).[1] However, despite standard treatment, approximately 10% of patients experience an IBTR within 10 years of diagnosis, associated with an increased risk of subsequent distant metastasis and death.[1 2] Patients with high-risk tumors may be recommended RT boost to eliminate residual microscopic tumor foci.[3] The most widely accepted boost indication is young age.[3] Furthermore, other characteristics of tumor aggressivity represent additional boost indications, although the definition varies between guidelines.[3 4] RT de-escalation has so far focused on low-risk tumors. However, recent data indicate significant prognostic heterogeneity among patients with high-risk tumors, for example, young individuals with estrogen receptor (ER)-negative tumors.[5] This is an area where immunological biomarkers show great potential.[5] In light of the above, we believe it is highly relevant to study the possibility of RT de-escalation in high-risk groups.

CD8+T cells are considered the primary effector cell of the antitumoral immune response[6 7] and react to protein products of mutated tumor genes (ie, neoantigens). T cells are regulated by the programmed cell death protein-1 (PD-1)/programmed death ligand-1 (PD-L1) pathway and other immune checkpoints.[8 9] Despite its inherent inhibitory effect on CD8+T cells, an active PD-1/PD-L1 pathway may correlate with an activated immune response and an improved

prognosis among aggressive subtypes.[10] Assessments of the PD-1/PD-L1 axis provide independent information in addition to tumor-infiltrating lymphocytes (TILs),[11] but it is unknown if this can be used to improve RT individualization. We have previously shown that high stromal TILs may be associated with a reduced risk of IBTR and decreased RT benefits.[12]

Histological grade has long been an important prognostic factor in breast cancer and primarily measures proliferation and dedifferentiation.[13] In a previous study, we found that a signature correlating strongly with histological grade could predict the prognostic effect of an activated immune infiltrate[14]—a characteristic we will henceforth refer to as immune responsiveness. Histological grade may thus represent tumor-intrinsic qualities that predict the biological implications of a local immune infiltrate. However, many tumors are classified as grade II, which does not provide useful clinical information.[15] Previous studies indicate that subtype, in part, can determine immune responsiveness.[6] Subtype correlates with proliferation, whose biological relevance is illustrated by the fact that it may explain most of the performance of prognostic breast cancer signatures.[16 17] Because the luminal B subtype exhibits significant heterogeneity regarding proliferation,[18] we do not believe that subtype alone is the optimal method to estimate immune responsiveness. This is supported by recent data indicating immunotherapy responsiveness among a subset of luminal B tumors.[19] For this reason, we chose to use histological grade as a hypothesized marker of immune responsiveness in this study.

This study aimed to investigate whether an integrated analysis of TILs, the PD-1/PD-L1 signaling pathway, and histological grade can identify immune-responsive tumors from a cohort dominated by luminal tumors and inform RT de-escalation. These biomarkers are already being evaluated in clinical practice, and an increased understanding of their interaction for determining RT benefit and immune responsiveness may improve the treatment of patients with breast cancer. Using our previously developed gene expression signature predicting immune responsiveness,[14] we also attempted to stratify grade II tumors into high-risk and low-risk groups with hypothesized different benefits of a local immune infiltrate. We hypothesized that high-risk tumors with an activated immune response could be downgraded in terms of locoregional treatment.

METHODS

Study population

Patients from the SweBCG91RT trial were analyzed.[20 21] In summary, 1178 patients with lymph node-negative (N0) stage I or IIA breast cancer were randomly assigned between 1991 and 1997 to BCS with or without whole-breast RT and followed for a median time of 15.2 years(online supplemental file 3) (figure 1). No patient had a positive surgical margin. Systemic adjuvant therapy

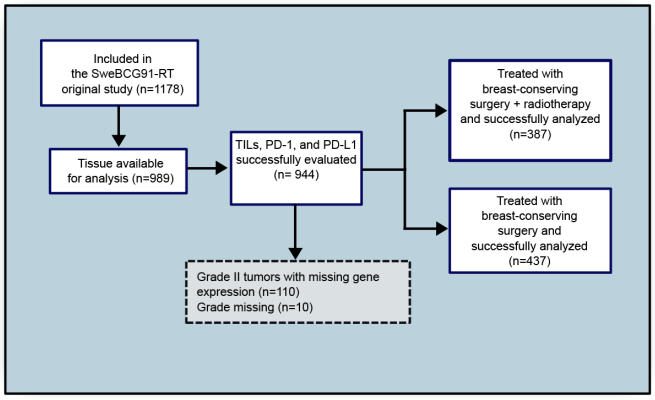

Figure 1 Consort diagram of included patients. Tumor blocks from patients included in the original SweBCG91RT trial were recollected. TILs and histological grade were scored on whole tissue sections and PD-1/PD-L1 were scored on TMAs. PD-1, programmed cell death protein-1; PD-L1, programmed death ligand-1; RT, radiotherapy; TILs, tumor-infiltrating lymphocytes; TMA, tissue microarray.

was given per regional guidelines at the time. In total, 7% of patients received endocrine treatment, 1% received chemotherapy, and 0.4% received both endocrine therapy and chemotherapy. Tumor blocks were recollected and tumor subtyping was performed according to the St Gallen International Breast Cancer Conference (2013) Expert Panel on tissue microarray (TMA) slides as described previously.[22] In short, tumors were classified as luminal A–like (ER-positive, progesterone receptor (PgR)-positive, human epidermal growth factor receptor 2 (HER2)-negative, and Ki-67 low), luminal B–like (ER-positive, PgR-negative or Ki-67 high, and HER2-negative), HER2-positive (HER2-positive, any ER and PgR status, any Ki-67) and triple-negative (ER-negative, PgR-negative, HER2-negative, and any Ki-67). Analyses were performed on treatment-naïve formalin-fixed paraffin-embedded (FFPE) tumor samples. Invasive carcinoma was histologically confirmed by a board-certified pathologist. Included patients did not differ from excluded patients except for histological grade and tumor size. Excluded patients had slightly smaller tumors of a lower histological grade (online supplemental table S1).

The original trial and follow-up study were conducted per the Declaration of Helsinki. Oral informed consent was obtained from all patients before performing human investigations for the original trial and this follow-up study, and was determined appropriate and approved by the Ethical Review Board.

Data sharing

Gene expression data has been deposited in the Gene Expression Omnibus under accession number GSE119295. Due to regulations of the ethical review board and laws related to patient privacy, all clinical information has not been made publicly available.

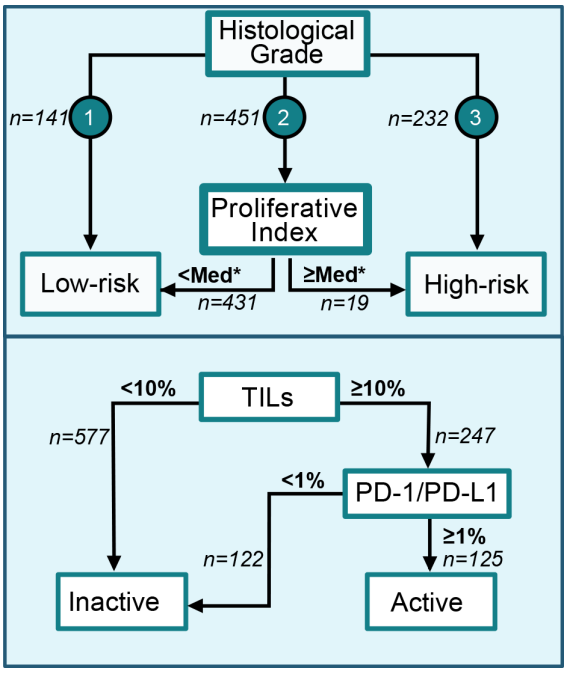

Figure 2 Flow charts for the classification of tumors into low-risk and high-risk tumor-intrinsic groups as well as of immune infiltrates as activated or inactivated/absent. *The median score of grade III tumors was used as the cut-off to classify grade II tumors as low-risk or high-risk. PD-1, programmed cell death protein-1; PD-L1, programmed death ligand-1; TILs, tumor-infiltrating lymphocytes.

Immunohistochemistry evaluations

Stromal TILs were evaluated on whole tissue H&E-stained sections as described previously.[12] In short, TILs were evaluated as semicontinuous values (0%, 1–9%, 10–49%, 50–74%, 75–100%) by two board-certified pathologists, who were blinded to the outcome, until consensus was reached.[12] Evaluations of PD-1 and PD-L1 were performed on TMAs by two board-certified pathologists using the Cell Marque (NAT105) and Ventana (SP142) antibodies. Two cores per marker were evaluated, and the highest value per marker was chosen, given that TMA evaluations of immune checkpoint proteins tend to underestimate the degree of positive staining.[23] Staining of ≥1% of lymphocytes was defined as positive, as this is the cut-off used in clinical practice to determine PD-L1 positivity[24] (an image of positive staining can be found in the online supplemental file 1). Staining protocols are included in the online supplemental file 1. We defined an activated immune infiltrate as TILs ≥10% and positive staining for at least one of PD-1 or PD-L1 (figure 2). We based this on previous literature indicating that TILs and immune checkpoint molecule expression provide independent information, complementing each other.[25] Consequently, combining TILs with checkpoint molecule expression measurements may allow for identifying the most immunogenic tumors compared with either marker alone.[26]

Tumor-intrinsic risk group assessment

We then divided patients into low-risk and high-risk groups depending on histological grade and the previously developed Proliferative Index signature.[14] Histological grade I was classified as low-risk and grade III as high-risk. In our previous study, Proliferative Index demonstrated a strong correlation with histological grade and proliferation, and could predict the immune responsiveness of tumors. We hypothesized that grade II tumors are heterogeneous and can be reclassified into high-risk or low-risk as previously suggested.[27] Most tumors of the SweBCG91RT cohort were previously classified as grade II. Since the literature indicates that an immune infiltrate's prognostic effect in low-risk, ER-dominated, cohorts is either absent or unfavorable, we hypothesized that the majority of grade II tumors should be classified as low-risk and not immune-responsive.[28–30] This hypothesis was further supported by the fact that the Proliferative Index of grade II tumors resembled grade I tumors more than grade III tumors (online supplemental figure S1). We, therefore, hypothesized that grade II tumors were more similar to grade I tumors regarding the biological implications of an immune infiltrate. To accurately reclassify grade II tumors based on their hypothesized immune responsiveness, we set the cut-off for high-risk grade II tumors at the median Proliferative Index of grade III tumors. The remainder of grade II tumors were classified as low-risk (figure 2). The high cut-off was further motivated by the fact that we did not want to dilute the hypothesized effect size of the high-risk group.

Statistical methods

Time to IBTR as the first event within 10 years from diagnosis was used as the primary endpoint. The aims were to analyze the interaction between an activated immune response and tumor-intrinsic risk group (high-risk or low-risk) on the risk of IBTR and its implications for the benefit from RT. A likelihood-ratio test between regression models with and without an interaction term was used to test the interaction effect. A p value<0.05 was considered significant. P values reported for other analyses, which were not part of the main hypothesis, were not adjusted for multiple hypothesis testing and should be interpreted with caution. Hazard ratios (HRs) with 95% confidence intervals (CIs) presented in tables and the results section were calculated with cause-specific Cox proportional hazards regression to reflect the biological effect of an activated immune infiltrate depending on tumor-intrinsic risk groups in the presence of competing risks. Other recurrences and deaths were considered competing risks for IBTR. Cumulative incidences were used to describe 10-year IBTR rates. Figures of cumulative incidences were created according to the method of Fine and Gray[31] and based on the Cox models of subhazards, producing subdistribution HRs. P values for differences in cumulative incidences between compared groups were denoted as P_{CIF} in the plots. Age, tumor size, ER status,

and RT were tested in univariable analysis and, if significant, included in multivariable analysis.

The proportional hazards assumption was checked using the Schoenfeld residuals. It was violated for histological grade and RT. Therefore, estimates for these variables should be regarded as the mean effect over the 10-year follow-up period. Due to the violation of the proportional hazards assumption, we also included analyses with a follow-up time of 5 years in the supplement (online supplemental tables S2–S4). The results of these analyses were similar to those presented in the main manuscript, and the proportional hazards assumption was not violated.

Stata V.17.0 was used for analysis (StataCorp. 2017, Stata: Release 17, Statistical Software, StataCorp).

RESULTS

Demographics

In total, 148 (15.4%) tumors were classified as grade I, 573 (59.8%) as grade II, and 237 (24.7%) as grade III. We calculated the previously developed signature, Proliferative Index, and centered and standardized the scores to have a mean of 0 and an SD of 1. We then used the Proliferative Index to classify grade II tumors as high-risk or low-risk (figure 2). Grade I tumors had a median Proliferative Index of −0.70, grade II tumors −0.43, and grade III tumors 1.03 (online supplemental figure S1). A total of 19 (3.3%) of the 573 grade II tumors had a Proliferative Index equal to or higher than the median of grade III tumors and were classified as high-risk.

In total, 139 (55.4%) of high-risk tumors had high TILs (≥10%), 62 (24.7%) had a high PD-1 expression (≥1%), and 101 (40.2%) had a high PD-L1 expression (≥1%) (table 1). A total of 96 (38.2%) tumors were classified as having an activated immune response (TILs ≥10% and PD-1 and/or PD-L1 ≥1%). A total of 75 (36.1%) high-risk tumors were ER negative, 232 (92.4%) tumors were of grade III, and 19 (7.6%) were of grade II (table 1, online supplemental table S5). Tumors with TILs ≥10% and PD-1/PD-L1 expression ≥1% generally had higher TILs than tumors with TILs ≥10% but without PD-1/PD-L1 expression (online supplemental table S6).

In the low-risk group, high TILs were seen among 108 tumors (18.8%), high PD-1 expression among 48 (8.4%) tumors, and high PD-L1 expression among 62 (10.8%) tumors (table 1). In total, 29 (5.1%) tumors were classified as having an activated immune response. Among low-risk tumors, 12 (2.3%) were ER-negative, 141 (24.6%) of grade I, and 432 (75.4%) of grade II.

Prognostic effect

In total, 17.2% (13.1–22.5) of patients in the high-risk group and 13.7% (11.1–16.8) of patients in the low-risk group developed an IBTR within 10 years. High-risk tumors with an active immune response had an IBTR rate of 8.4% (4.3–16.1), while high-risk tumors without an active immune infiltrate had an IBTR rate of 22.8% (16.9–30.2). Among high-risk tumors, an activated immune infiltrate was associated with a reduced risk of IBTR in univariable (HR 0.34, 95% CI 0.16 to 0.73, p=0.006) and multivariable (HR 0.33, 95% CI 0.15 to 0.72, p=0.005) analysis (table 2).

Low-risk tumors with an activated immune infiltrate had a 10-year IBTR rate of 20.9% (10.0–40.7) compared with an IBTR rate of 13.3% (10.7–16.5) among low-risk tumors without an activated immune infiltrate. No significant difference in risk IBTR among low-risk tumors was seen for an activated immune infiltrate (univariable: 2.0, 95% CI 0.87 to 4.6, p=0.100, multivariable: HR 1.8, 95% CI 0.79 to 4.2, p=0.159) compared with not having an activated immune infiltrate (HR 1.0) (table 2. The interaction between immunological activity and risk group was significant in univariable (p=0.005) and multivariable (p=0.007) analysis (table 3).

Benefit from RT

A non-significant benefit from RT was seen among high-risk tumors with an activated immune infiltrate (HR 0.34, 95% CI 0.07 to 1.67, p=0.182), while a significant benefit was observed among high-risk tumors without an activated immune infiltrate (HR 0.40, 95% CI 0.18 to 0.88, p=0.022). Among low-risk tumors with an activated immune infiltrate, the estimates for RT benefit (HR 0.40, 95% CI 0.05 to 3.44, p=0.403) were similar to those of low-risk tumors without an activated immune infiltrate (HR 0.42, 95% CI 0.25 to 0.69, p=0.001).

Figure 3 illustrates the cumulative incidences depending on RT, immune activation, and tumor-intrinsic risk group. High-risk tumors with an activated immune response had a 10-year incidence of IBTR of 12.1% (5.6–25.0) without RT and 4.4% (1.1–16.3) with RT. This can be contrasted against high-risk tumors with an absent immune response, where the 10-year incidence of IBTR was 29.6% (21.4–40.2) without RT and 12.8% (6.6–23.9) with RT. Low-risk tumors with an activated immune response had a 10-year IBTR incidence of 25.0% (11.2–50.0) without RT and 11.1% (1.6–56.7) with RT, while low-risk tumors without an activated immune infiltrate had a 10-year incidence of IBTR of 18.1% (14.0–23.3) without RT and 8.4% (5.6–12.5) with RT.

Exploratory analyses

As a post hoc exploratory analysis, we compared the high-risk groups with TILs 10–49% and 50–100% to investigate a potential dose-response relationship. Unirradiated patients with TILs 10–49% had a 10-year cumulative IBTR incidence of 15% (0.07–0.29). Unirradiated patients with TILs 50–100% had a lower, but not significantly different, cumulative IBTR incidence of 13% (0.05–0.31) (online supplemental figure S2).

Finally, to verify the stability of the results, we re-ran the main analyses excluding patients treated with systemic therapy. The findings remained stable (online supplemental tables S7 and S8).

Table 1 Demographics of included patients

Variables	Low-risk group			High-risk group		
	No RT	RT	Total	No RT	RT	Total
TILs						
Low	240 (81.4%)	225 (80.9%)	465 (81.2%)	63 (44.4%)	49 (45.0%)	112 (44.6%)
High	55 (18.6%)	53 (19.1%)	108 (18.8%)	79 (55.6%)	60 (55.0%)	139 (55.4%)
PD-1						
Low	267 (90.5%)	258 (92.8%)	525 (91.6%)	109 (76.8%)	80 (73.4%)	189 (75.3%)
High	28 (9.5%)	20 (7.2%)	48 (8.4%)	33 (23.2%)	29 (26.6%)	62 (24.7%)
PD-L1						
Low	255 (86.4%)	256 (92.1%)	511 (89.2%)	89 (62.7%)	61 (56.0%)	150 (59.8%)
High	40 (13.6%)	22 (7.9%)	62 (10.8%)	53 (37.3%)	48 (44.0%)	101 (40.2%)
Immune activation						
Active*	20 (6.8%)	9 (3.2%)	29 (5.1%)	50 (35.2%)	46 (42.2%)	96 (38.2%)
Inactive/absent†	275 (93.2%)	269 (96.8%)	544 (94.9%)	92 (64.8%)	63 (57.8%)	155 (61.8%)
Subtype						
HER2-positive‡	6 (2.2%)	9 (3.5%)	15 (2.8%)	19 (16.2%)	19 (21.6%)	38 (18.5%)
Luminal A	193 (71%)	175 (67.8%)	368 (69.4%)	26 (22.2%)	18 (20.5%)	44 (21.5%)
Luminal B	70 (25.7%)	72 (27.9%)	142 (26.8%)	37 (31.6%)	27 (30.7%)	64 (31.2%)
Triple-negative	3 (1.1%)	2 (0.8%)	5 (0.9%)	35 (29.9%)	24 (27.3%)	59 (28.8%)
ER status						
Negative	7 (2.6%)	5 (1.9%)	12 (2.3%)	42 (35.3%)	33 (37.1%)	75 (36.1%)
Positive	266 (97.4%)	254 (98.1%)	520 (97.7%)	77 (64.7%)	56 (62.9%)	133 (63.9%)
PgR status						
Negative	41 (15.0%)	48 (18.5%)	89 (16.7%)	59 (49.6%)	48 (53.9%)	107 (51.4%)
Positive	232 (85.0%)	211 (81.5%)	443 (83.3%)	60 (50.4%)	41 (46.1%)	101 (48.6%)
Histological grade						
Grade I	68 (23.1%)	73 (26.3%)	141 (24.6%)	0 (0%)	0 (0%)	0 (0%)
Grade II	227 (76.9%)	205 (73.7%)	432 (75.4%)	10 (7.0%)	9 (8.3%)	19 (7.6%)
Grade III	0 (0%)	0 (0%)	0 (0%)	132 (93.0%)	100 (91.7%)	232 (92.4%)
Endocrine therapy						
No hormone therapy	254 (92.7%)	249 (95.8%)	503 (94.2%)	105 (88.2%)	80 (88.9%)	185 (88.5%)
Hormone therapy	20 (7.3%)	11 (4.2%)	31 (5.8%)	14 (11.8%)	10 (11.1%)	24 (11.5%)
Chemotherapy						
No chemotherapy	274 (100%)	259 (99.6%)	533 (99.8%)	113 (95.0%)	87 (96.7%)	200 (95.7%)
Chemotherapy	0 (0%)	1 (0.4%)	1 (0.2%)	6 (5.0%)	3 (3.3%)	9 (4.3%)
IBTR within 5 years§						
No IBTR	240 (81.4%)	255 (91.7%)	495 (86.4%)	96 (67.6%)	86 (78.9%)	182 (72.5%)
IBTR	37 (12.5%)	7 (2.5%)	44 (7.7%)	27 (19.0%)	8 (7.3%)	35 (13.9%)
Censored	18 (6.1%)	16 (5.8%)	34 (5.9%)	19 (13.4%)	15 (13.8%)	34 (13.5%)
IBTR within 10 years§						
No IBTR	206 (69.8%)	221 (79.5%)	427 (74.5%)	80 (56.3%)	69 (63.3%)	149 (59.4%)
IBTR	54 (18.3%)	23 (8.3%)	77 (13.4%)	33 (23.2%)	10 (9.2%)	43 (17.1%)
Censored	35 (11.9%)	34 (12.2%)	69 (12.0%)	29 (20.4%)	30 (27.5%)	59 (23.5%)

*Defined as TILs ≥10% and PD-L1 and/or PD-1 ≥1%.
†Defined as TILs <10% or TILs ≥10% but PD-L1 and PD-1 <1%.
‡Includes both ER-positive and ER-negative tumors.
§Reported as absolute frequencies rather than cumulative incidences.
ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IBTR, ipsilateral breast tumor recurrence; PD-1, programmed cell death protein-1 ; PD-L1, programmed death ligand-1 ; PgR, progesterone receptor; RT, radiotherapy; TILs, tumor-infiltrating lymphocytes.

Table 2 Cox proportional hazard rate regression. Ten-year follow-up of ipsilateral breast tumor recurrence (IBTR) among low-risk and high-risk patients

Variable	Low-risk group (n=573)				High-risk group (n=251)			
	# of IBTR/ # of patients	Univariable Cox regression HR (95% CI)	P value	Multivariable Cox regression HR (95% CI)	P value	# of IBTR/ # of patients	Univariable Cox regression HR (95% CI)	P value	Multivariable Cox regression HR (95% CI)	P value
Immune system										
Not activated	71/544	1.0		1.0		35/155	1.0		1.0	
Activated	6/29	2.0 (0.87 to 4.6)	0.100	1.8 (0.79 to 4.2)	0.159	8/96	0.34 (0.16 to 0.73)	0.006	0.33 (0.15 to 0.72)	0.005
Age (cont.)	77/573	0.98 (0.95 to 1.0)	0.102	–	–	43/251	0.97 (0.94 to 0.99)	0.019	0.97 (0.94 to 0.99)	0.016
Tumor size (cont.)	71/530	1.0 (0.96 to 1.1)	0.739	–	–	39/208	0.98 (0.93 to 1.0)	0.518	–	–
ER										
Negative	4/9	1.0		1.0		11/85	1.0			
Positive	73/561	0.21 (0.08 to 0.56)	0.002	0.17 (0.06 to 0.46)	0.001	32/165	1.4 (0.72 to 2.8)	0.302	–	–
RT										
No	54/295	1.0		1.0		33/142	1.0		1.0	
Yes	23/278	0.41 (0.25 to 0.67)	<0.001	0.40 (0.25 to 0.66)	<0.001	10/109	0.36 (0.18 to 0.74)	0.005	0.42 (0.20 to 0.85)	0.017

ER, estrogen receptor; IBTR, ipsilateral breast tumor recurrence; RT, radiotherapy.

DISCUSSION

The integration of immunological and tumor-intrinsic factors enables the successful stratification of high-risk tumors regarding the risk of IBTR. The present study shows that this can generally be achieved with variables already used in the clinic and that aggressive tumors, including luminal subtypes, with an active immune response, have a low risk of IBTR even without RT boost and systemic therapy. High-risk tumors with activated immune infiltrates had the lowest rates of IBTR, highlighting the possibility to de-intensify locoregional treatment.

Research on biological predictors to inform RT de-escalation is ongoing. The recently published POLAR classifier may identify ER-positive HER2-negative tumors suited for RT omission.[32] Genes associated with proliferation were associated with an increased risk of locoregional recurrence. The current study highlights a parallel de-escalation pathway on the opposite side of the proliferation spectrum, where traditionally regarded high-risk tumors, irrespective of ER status, can be downgraded if they benefit from an activated antitumor immune response. Patients with high-risk tumors with an activated immune infiltrate had a relatively low risk of IBTR unirradiated (12.1%) and irradiated (4.4%), despite standard RT (ie, without an RT boost) and a low frequency of systemic therapy. These tumors may have a delayed local and systemic dissemination preoperatively and inhibited regrowth of postoperative residual disease, reducing the need for RT treatment. With modern systemic treatment, the 10-year incidence of IBTR may be below 10% without RT. The findings align with another recent study showing that young patients with triple-negative breast cancer and high TILs have a surprisingly good prognosis without adjuvant therapy.[5] Immune-responsive tumors with very high TIL levels (eg, ≥50%) may represent an RT omission group, while moderately increased TILs (eg, 10–49%) could justify RT boost omission. Although we hypothesize that low-risk tumors are best stratified for treatment de-escalation using proliferation measurements, the role of the immune response among these is not fully understood. We and others have previously shown that global measures of immune activation confer a favorable prognosis only among high-risk tumors.[14 33 34] However, it cannot be excluded that activation of certain immune response subcomponents may still benefit low-risk tumors. For example, the humoral immune system may reduce the recurrence risk in luminal tumors,[35] which conforms with findings of B-cell-related genes in POLAR predicting a favorable prognosis.[32]

We have previously shown that integrating tumor-intrinsic factors in the assessment of immunological biomarkers can improve the identification of high-risk tumors with different needs for RT. [14] CD8+T cells, the primary effector cell of antitumor immunity, recognize and are activated by neoantigens generated by tumor mutations.[7] Therefore, tumor-intrinsic factors that correlate with proliferation and tumor mutational burden (TMB) may inform the likelihood that

Table 3 Cox proportional hazard rate regression. Ten-year follow-up of ipsilateral breast tumor recurrence (IBTR)

Variables	No. of IBTRs/no. of patients	Univariable Cox regression		Multivariable Cox regression	
		HR (95% CI)	P value	HR (95% CI)	P value
Combination of immune group and risk group					
Not activated, low risk	71/544	1.0		1.0	
Not activated, high risk	35/155	2.1 (1.4 to 3.1)	<0.001	1.9 (1.3 to 2.9)	0.002
Activated, low risk	6/29	2.0 (0.87 to 4.6)	0.105	1.8 (0.76 to 4.0)	0.189
Activated, high risk	8/96	0.68 (0.33 to 1.4)	0.306	0.63 (0.30 to 1.3)	0.217
Interaction			0.005*		0.007*
Age (cont.)	120/824	0.97 (0.95 to 0.99)	0.005	0.97 (0.96 to 0.99)	0.003
Tumor size (cont.)	110/738	1.01 (0.97 to 1.04)	0.761	–	
ER status					
Negative	15/94	1.0		–	
Positive	105/726	0.78 (0.46 to 1.35)	0.376	–	
RT					
No	87/437	1.0		1.0	
Yes	33/387	0.39 (0.26 to 0.58)	<0.001	0.41 (0.27 to 0.61)	<0.001

*Likelihood-ratio test.
ER, estrogen receptor; IBTR, ipsilateral breast tumor recurrence; RT, radiotherapy.

an immune infiltrate represents an active antitumoral immune response.[34] Histological grade correlates with proliferation and TMB,[36] and we hypothesized that histological grade might capture tumor-intrinsic qualities necessary to understand the biological influence of an immune infiltrate. In the SweBCG91RT cohort, PD-1 and/or PD-L1 were expressed by the majority of high-risk tumors with high TILs. Conversely, high TILs were less frequently associated with PD-1/PD-L1 expression among low-risk tumors, indicating that an immune infiltrate in these tumors has other biological implications. This is supported by studies showing an absent or unfavorable prognostic effect of immune infiltrates in low-risk tumors.[28–30]

Despite the overwhelming focus on triple-negative and HER2-positive subtypes in TILs research, most tumors with TILs are ER-positive.[11] However, the lack of understanding of how TILs influence tumor progression among ER-positive tumors has prevented TILs from being used as a biomarker in this group.[11] A better understanding may enable the implementation of immunotherapy on a subset of immunogenic ER-positive tumors.[11] We found that the majority of tumors classified as high-risk, and deriving a significant benefit from an activated immune infiltrate, were ER-positive (63.9%), echoing the unmet potential for using TILs as a biomarker among these tumors.[11] The International Immuno-Oncology Biomarker Working Group highlights the need for more research on TILs among ER-positive subtypes, stratifying analyses by luminal A and luminal B.[11] However, our results indicate that there may exist heterogeneity within these subtypes, as all subtypes were relatively equally represented in the high-risk group. Our findings add a layer of complexity to previous observations[37] by suggesting that it may not be subtype, but instead characteristics that can in part be approximated by subtype, that predict the biological influence of an immune infiltrate. These findings align with a previous study where luminal B tumors with aggressive tumor characteristics demonstrated immunotherapy responsiveness.[19] We believe additional measures of tumor aggressiveness, such as histological grade or proliferation, are needed to accurately predict the implications of an immune infiltrate, particularly in the case of luminal B tumors, where the degree of proliferation can vary considerably.[18] It remains to be determined if tumor-intrinsic characteristics predict immune responsiveness also in cohorts dominated by non-luminal subtypes.

Assessments of the PD-1/PD-L1 pathway are today used as biomarkers for immunotherapy in metastatic triple-negative breast cancer.[38] Expression is associated with an improved prognosis,[39] despite the inherent immunosuppressive effects, likely due to its association with an active immune response. PD-1 is expressed on activated T cells,[40] and PD-L1, expressed by a wide range of cells, for example, T-regulatory cells and tumor cells, is upregulated by inflammatory signaling.[41] Furthermore, measurements of the PD-1/PD-L1 pathway provide independent information in addition to TILs.[26] For this reason, we used high TILs combined with the expression of PD-1 or PD-L1 to characterize an active immune response.

We used histological grade and a gene expression-based proliferation signature as tumor-intrinsic predictors of

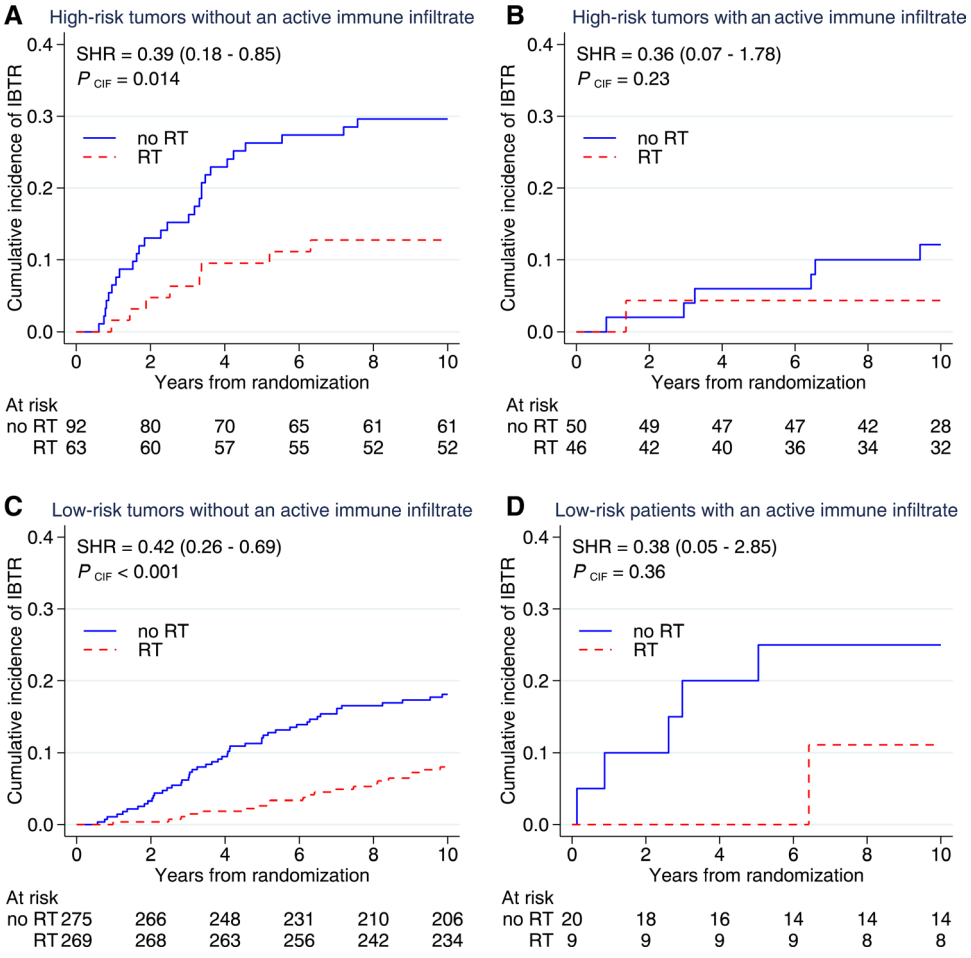

Figure 3 Cumulative incidences among high-risk and low-risk tumors with and without an activated immune response. High-risk tumors were defined as histological grade III or histological grade II with a high Proliferative Index. An activated immune response was defined as tumor-infiltrating lymphocytes ≥10% and ≥1% of lymphocytes positive for programmed cell death protein-1 and/or programmed death ligand-1. IBTR, ipsilateral breast tumor recurrence; RT, radiotherapy; SHR, subdistribution hazard ratio; CIF, cumulative incidence function.

immune responsiveness.[42] However, additional tumor characteristics should be considered. One such factor is HER2 status, which has emerged as a key biomarker in breast cancer. HER2-positive tumors are considered immunogenic, and anti-HER2 therapy functions partly by inducing an antitumoral immune response.[42 43] Unfortunately, due to the low number of HER2-positive tumors and lack of anti-HER2 therapy, we did not try to answer whether HER2 positivity should be included as a variable predictive of immune responsiveness. Therefore, future studies should investigate whether HER2 positivity and additional tumor-intrinsic characteristics, such as TMB, provide independent information beyond histological grade and tumor proliferation regarding immune responsiveness.

There are several weaknesses in the present study. First, our question involved post hoc analyses of subgroups, which reduces the power and should be viewed as hypothesis-generating. The low-risk group with an active immune infiltrate was small, why findings pertaining to this group should be interpreted cautiously. Second, many patients would have received a different therapy regimen had they been diagnosed today. No patients in the SweBCG91RT study received an RT boost, although some of them would be recommended a boost in the current situation. In addition, few patients received systemic treatment, which would likely have significantly reduced the risk of IBTR.[44] Furthermore, systemic anti-HER2 therapy and chemotherapy treatment would probably have produced a differential benefit for patients, with highly proliferative immunogenic tumors showing the best response.[45 46] While the above limits the generalizability of our findings, it also indicates that modern treatment would preferentially have reduced the risk of IBTR in the high-risk immunogenic group, further supporting de-escalation of RT as a valid strategy for these patients. Nevertheless, our findings apply primarily to a setting free of adjuvant systemic therapy. The high cut-off used to classify grade II tumors as high-risk resulted in only a minority of these tumors being classified as such and did not allow for thoroughly investigating immune responsiveness along the spectrum of tumor aggressiveness among grade II tumors. We used this high cut-off based on the hypothesis that most grade II tumors should be classified as low-risk

and to avoid dilution of the hypothesized effect size of the high-risk group. We did not test additional cut-offs and cannot determine the proportion of grade II tumors likely to benefit from an immune infiltrate. This should be investigated in future studies. However, the finding that grade II tumors resemble grade I tumors more than grade III tumors in terms of a gene expression signature designed to measure immune responsiveness indicates that most should be classified as low-risk. Finally, the use of TMAs may miss tumor heterogeneity. Previous studies have shown that around three TMAs may be sufficient to categorize a tumor as having high or low TILs.[47] Since we used four TMAs to assess the activity of the PD-1/PD-L1 axis, we believe the risk of missing tumor heterogeneity is reduced, although not eliminated.

There is a large variation in analytical sensitivity between different PD-L1 immunohistochemistry (IHC) assays, with SP142, used in the present study, shown to have poor sensitivity.[48] Consequently, some tumors classified as PD-L1 negative were likely false negatives, indicating that findings should be interpreted cautiously. The optimal IHC assay identifying immunogenic tumors would preferably have a higher sensitivity than SP142. Furthermore, a potential added value to TILs of additional immunological markers, such as PD-1/PD-L1 expression,[25] may be partly or entirely explained by an association with even higher TILs. Therefore, assessing TILs as a continuous variable on whole sections may be a sufficiently robust measurement to identify tumors with different immune activation degrees and tailor therapy accordingly.

In conclusion, high-risk tumors with an activated immune infiltrate have a surprisingly good prognosis in terms of local recurrences. The risk reduction regarding IBTR conferred by an activated immune infiltrate among these tumors may be comparable to treatment with RT. Therefore, we hypothesize that these patients do well with the de-escalation of RT treatment. Our findings likely apply to low-risk early breast cancer cohorts dominated by ER-positive tumors.

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Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by Regional Ethical Review Board of Western Sweden (approval numbers 2010/127 and 2015/548). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. Gene expression data from the SweBCG91RT cohort has been made available in the Gene Expression Omnibus database (GSE119295). However, due to regulations of the ethical review board and of laws related to patient privacy, all clinical information has not been made publicly available. The IHC data used in this study is/are available from the corresponding author upon reasonable request.

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Supplement

Table of contents

Figure S1. Association of Proliferative Index with histological grade in the SweBCG91RT cohort

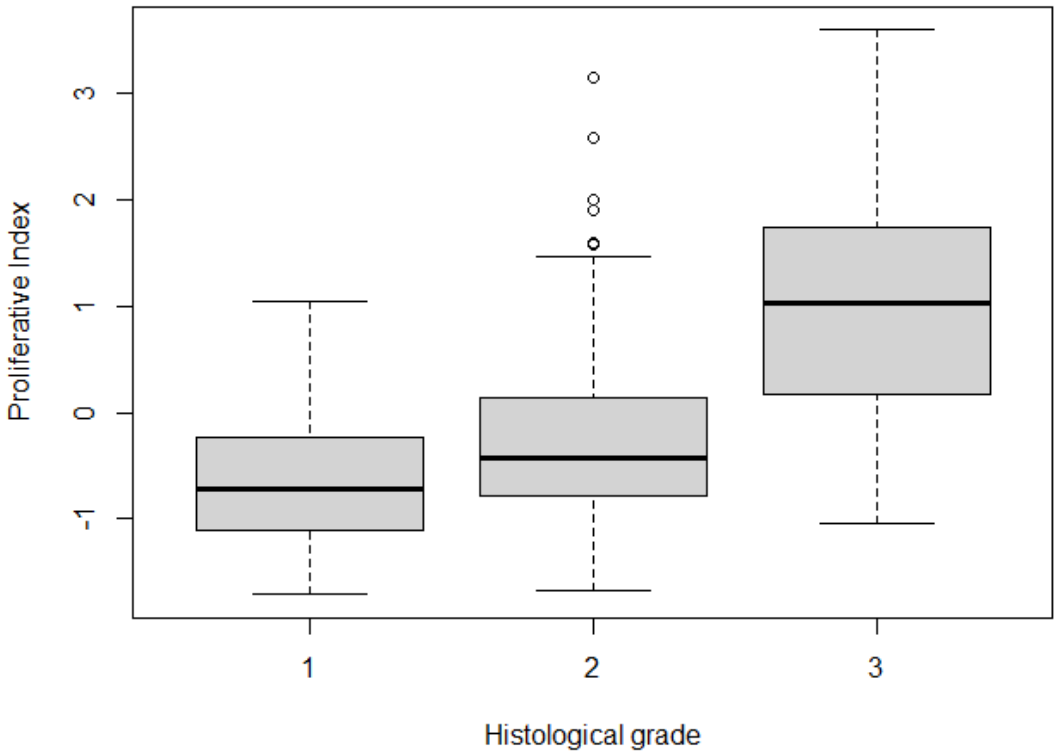
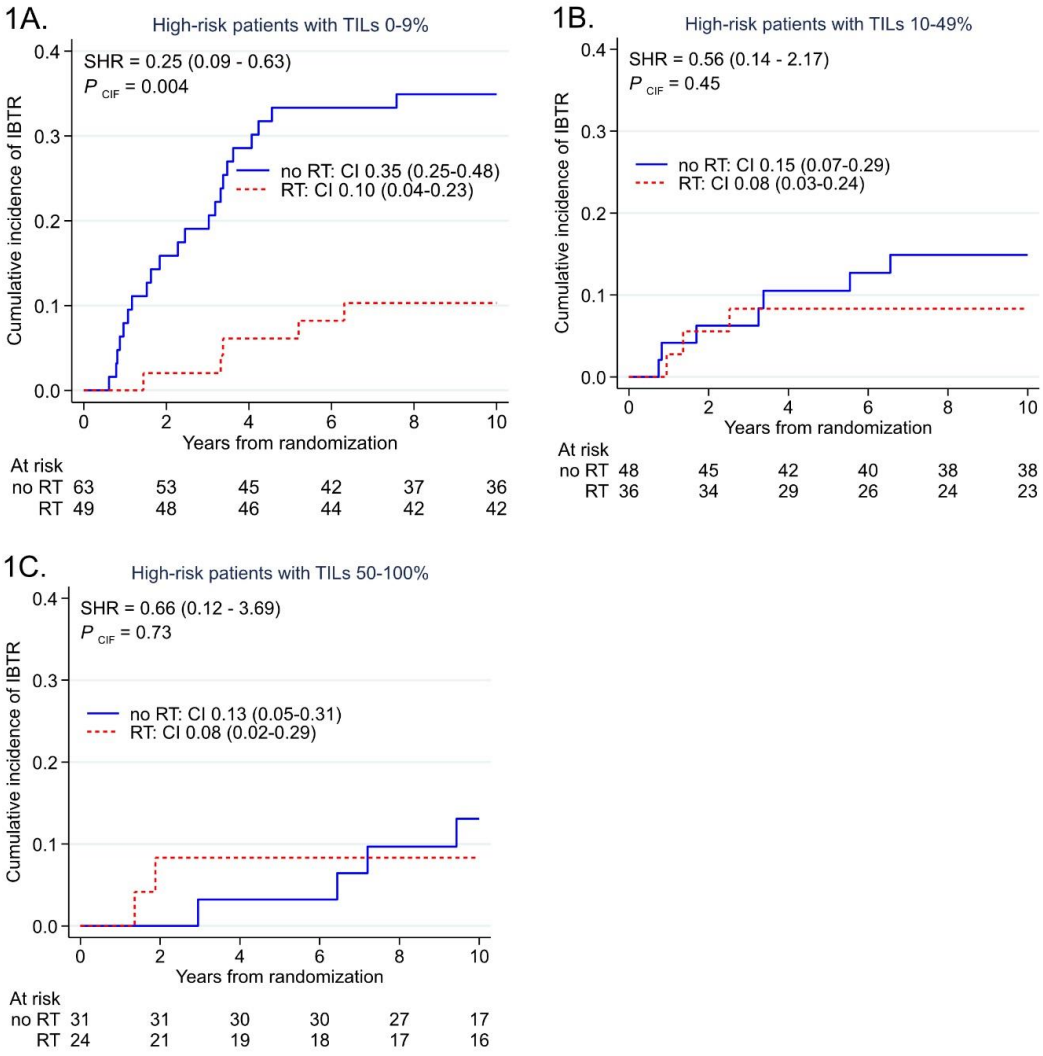


Figure S2. Cumulative incidence of IBTR in the high-risk group with increasing levels of TILs



RT= Radiotherapy. TILs= Tumor-infiltrating lymphocytes. SHR= Subhazard ratio for RT versus no RT. CI= Cumulative incidence.

Table S1. Comparison of included versus excluded patients

Variables	Included	Excluded	P
Age median	59	61	
Age mean	58.47	59.50	0.060
Age min	31	32	
Age max	78	78	
RT treated no	437 (53%)	178 (49%)	
RT treated yes	387 (47%)	185 (51%)	0.21
1-10 mm	183 (22%)	126 (35%)	
11-15 mm	419 (51%)	162 (45%)	
16-20 mm	95 (12%)	30 (8%)	
>20 mm	122 (15%)	41 (11%)	<0.001
Size missing	5	4	
Histological grade I	141 (17%)	7 (5%)	
Histological grade II	451 (55%)	122 (91%)	
Histological grade III	232 (28%)	5 (4%)	0.013
Histological grade missing	0	229	
Systemic treatment yes	681 (92%)	336 (93%)	0.64
Systemic treatment no	62 (8%)	27 (7%)	

Table S2. Cox proportional hazard rate regression. 5-year follow-up of local recurrence (IBTR).

Variable	# of IBTR/ # of patients	Univariable Cox regression		Multivariable Cox regression	
		HR (95% CI)	P	HR (95% CI)	P
Combination of Immune system and Risk group					
Not activated, low risk	40/544	1.0		1.0	
Not activated, high risk	30/155	3.0 (1.9-4.9)	<0.001	2.7 (1.7-4.4)	<0.001
Activated, low risk	4/29	2.2 (0.81-6.3)	0.121	1.8 (0.66-5.2)	0.242
Activated, high risk	5/96	0.75 (0.30-1.9)	0.553	0.69 (0.27-1.8)	0.439
Interaction			0.005*		0.010*
Age (cont.)	79/824	0.97 (0.95-0.99)	0.014	0.97 (0.95-0.99)	0.013
Tumor size (cont.)	71/738	1.02 (0.98-1.06)	0.380	-	
ER					
Negative	12/94	1.0		-	
Positive	67/726	0.63 (0.34-1.17)	0.147	-	
RT					
No	64/437	1.0		1.0	
Yes	15/387	0.25 (0.14-0.44)	<0.001	0.27 (0.15-0.47)	<0.001

*Likelihood-ratio test.

Table S3. Cox proportional hazard rate regression. 5-year follow-up of local recurrence (IBTR) among low risk-patients (n=573)

Variable	# of IBTR/ # of patients	Univariable Cox regression		Multivariable Cox regression	
		HR (95% CI)	P	HR (95% CI)	P
Immune system					
Not activated	40/544	1.0		1.0	
Activated	4/29	2.3 (0.81-6.3)	0.121	1.9 (0.69-5.4)	0.210
Age (cont.)	44/573	0.99 (0.96-1.0)	0.582	-	-
Tumor size (cont.)		1.0 (0.95-1.1)	0.732	-	-
ER					
Negative	4/9	1.0		1.0	
Positive	40/561	0.12 (0.04-0.33)	<0.001	0.09 (0.03-0.25)	<0.001
RT*					
No	37/295	1.0		1.0	
Yes	7/278	0.19 (0.08-0.42)	<0.001	0.18 (0.08-0.41)	<0.001

Table S4.

Cox proportional hazard rate regression. 5-year follow-up of local recurrence (IBTR) among high-risk patients (n=251)

Variable	# of IBTR/ # of patients	Univariable Cox regression		Multivariable Cox regression	
		HR (95% CI)	P	HR (95% CI)	P
Immune system					
Not activated	30/155	1.0		1.0	
Activated	5/96	0.25 (0.10-0.65)	0.005	0.25 (0.10-0.64)	0.004
Age (cont.)	35/251	0.95 (0.92-0.99)	0.006	0.96 (0.93-0.99)	0.005
Tumor size (cont.)	31/208	1.0 (0.94-1.1)	0.908	-	-
ER					
Negative	8/85	1.0			
Positive	27/165	1.6 (9.75-3.64)	0.212	-	-
RT*					
No	27/142	1.0		1.0	
Yes	8/109	0.36 (0.16-0.79)	0.011	0.44 (0.20-0.97)	0.041

Table S5. Estrogen receptor expression levels in the high- and low-risk groups

Risk group (column percent/row percent)	**0%**	**1-9%**	**10-49**	**50-74%**	**75-100%**	**P***
Low-risk	12 (13.6%/2.2%)	4 (80%/0.7%)	3 (37.5%/0.6%)	5 (62.5%/0.9%)	514 (80.9%/95.5%)	
High-risk	76 (86.4%/36.9%)	1 (20%/0.5%)	5 (62.5%/2.4%)	3 (37.5%/1.5%)	121 (19.1%/58.7%)	<0.001

*Fisher's exact test

More than half of the tumors in the high-risk category demonstrated a high estrogen receptor expression. Around one in five (19.1%) strongly ER-positive tumors may be hypothesized to be immune responsive based on this data, which may indicate that subtype alone is insufficient to identify these tumors.

Table S6. Levels of TILs among patients with TILs ≥10% with and without PD-1/PD-L1 expression

Groups		TILs 10-49%	TILs 50-74%	TILs 75-100%	P*
PD-1/PD-L1 expression	No	97 (89.0%)	10 (9.2%)	2 (1.8%)	<0.001
	Yes	66 (58.9%)	29 (25.9%)	17 (15.2%)	

*Fisher's exact test

PD-1/PD-L1 expression was defined as the expression of PD-1 or PD-L1 in ≥1% of lymphocytes in at least one TMA. Among tumors with TILs ≥10%, PD-1/PD-L1 expression was associated with higher TILs levels

Table S7. Cox proportional hazard rate regression of ipsilateral breast tumor recurrence (IBTR) with

			Univariable Cox regression		Multivariable Cox regression	
Variables		**No. of IBTRs/No. of patients**	**HR (95% CI)**	**P**	**HR (95% CI)**	**P**
Combination of immune group and risk group						
	Not activated, low risk	70/512	1.0		1.0	
	Not activated, high risk	33/132	2.2 (1.5-3.4)	<0.001	2.1 (1.4-3.2)	<0.001
	Activated, low risk	6/27	2.1 (0.91-4.8)	0.080	1.9 (0.81-4.3)	0.144
	Activated, high risk	7/79	0.69 (0.32-1.5)	0.349	0.65 (0.30-1.4)	0.285
	Interaction			0.002*		0.005*
Age (cont.)		116/750	0.97 (0.95-0.99)	0.005	0.97 (0.96-0.99)	0.005
Tumor size (cont.)		106/676	1.02 (0.98-1.06)	0.403	-	
ER status	Negative	13/72	1.0		-	
	Positive	103/674	0.72 (0.40-1.28)	0.259	-	
RT[#]	No	85/391	1.0		1.0	
	Yes	31/359	0.36 (0.24-0.54)	<0.001	0.38 (0.25-0.57)	<0.001

10-year follow-up excluding patients with systemic treatment (n=74)

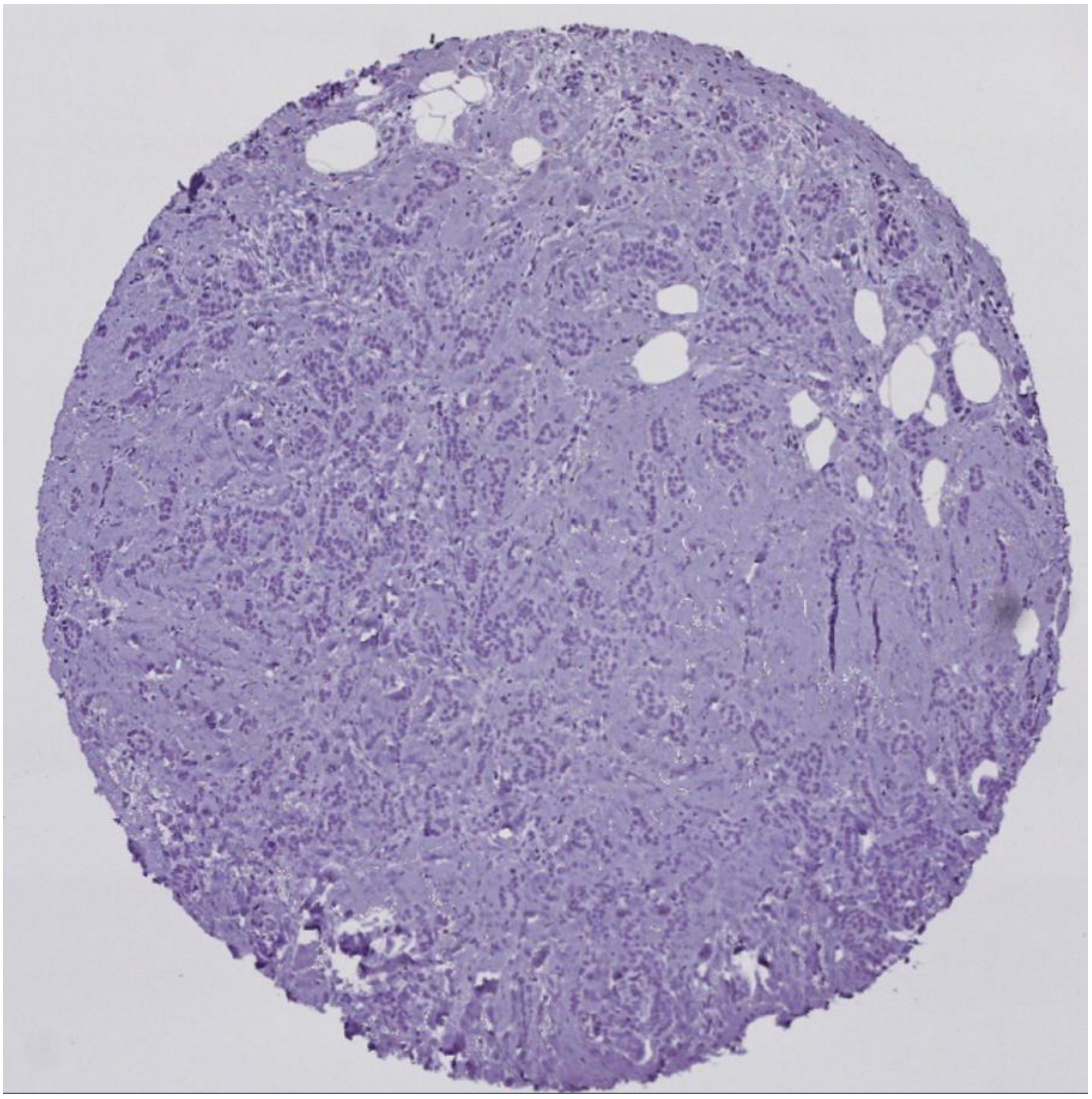
*Likelihood-ratio test.

Table S8. Cox proportional hazard rate regression of ipsilateral breast tumor recurrence (IBTR) 10-year follow-up among low-risk- and high-risk patients excluding patients with systemic treatment (n=74)

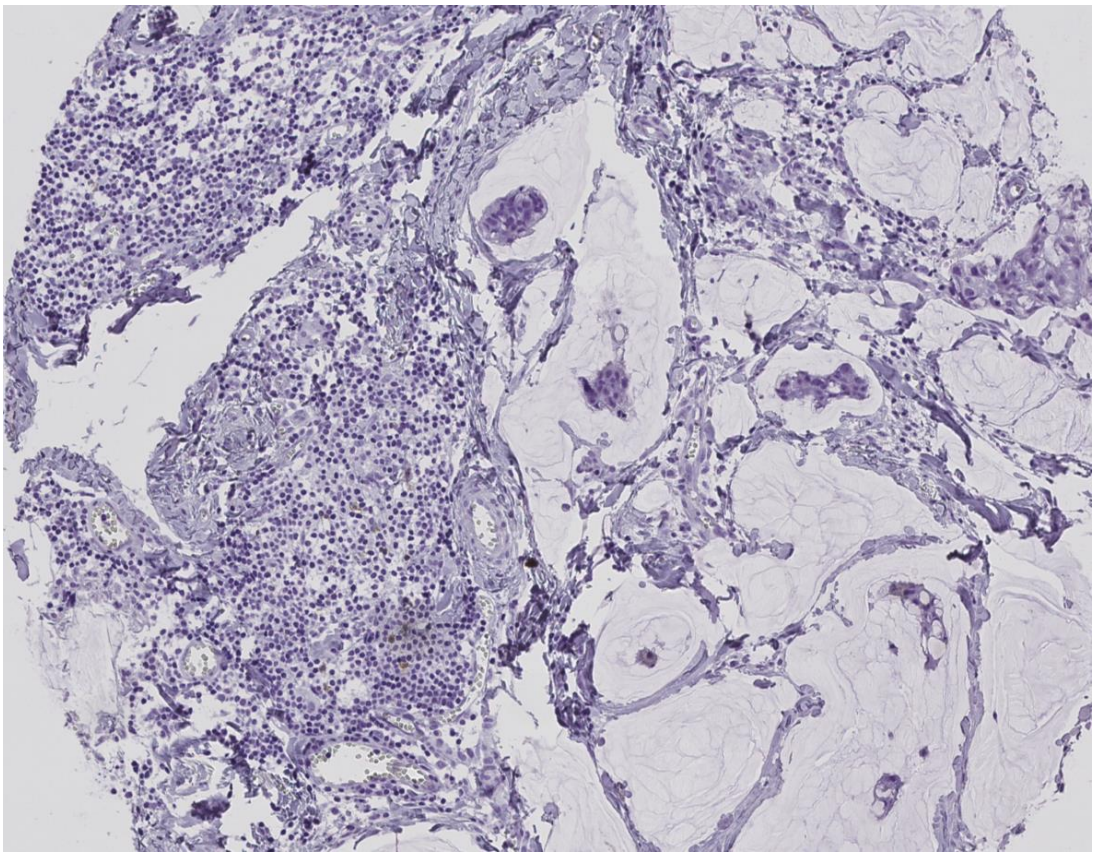
	Low-risk group (n=539)					High-risk group (n=211)				
	# of IBTR/	Univariable Cox regression		Multivariable Cox regression		# of IBTR/	Univariable Cox regression		Multivariable Cox regression	
Variable	# of patients	HR (95% CI)	P	HR (95% CI)	P	# of patients	HR (95% CI)	P	HR (95% CI)	P
Immune system										
Not activated	70/512	1.0		1.0		33/132	1.0		1.0	
Activated	6/27	2.1 (0.93-4.9)	0.075	2.0 (0.85-4.5)	0.115	7/79	0.32 (0.14-0.72)	0.006	0.32 (0.14-0.74)	0.007
Age (cont.)	76/539	0.98 (0.96-1.0)	0.119	-	-	40/211	0.96 (0.94-0.99)	0.018	0.97 (0.94-1.0)	0.021
Tumor size (cont.)	70/498	1.0 (0.97-1.1)	0.378	-	-	36/178	0.99 (0.93-1.1)	0.730	-	-
ER										
Negative	3/8	1.0		1.0		10/64	1.0			
Positive	73/528	0.29 (0.09-0.91)	0.034	0.23 (0.07-0.73)	0.012	30/146	1.2 (0.60-2.5)	0.580	-	-
RT[#]										
No	53/274	1.0		1.0		32/117	1.0		1.0	
Yes	23/265	0.41 (0.25-0.66)	<0.001	0.40 (0.25-0.66)	<0.001	8/94	0.29 (0.13-0.62)	0.002	0.32 (0.15-0.69)	0.004

Stainings of PD-1/PD-L1

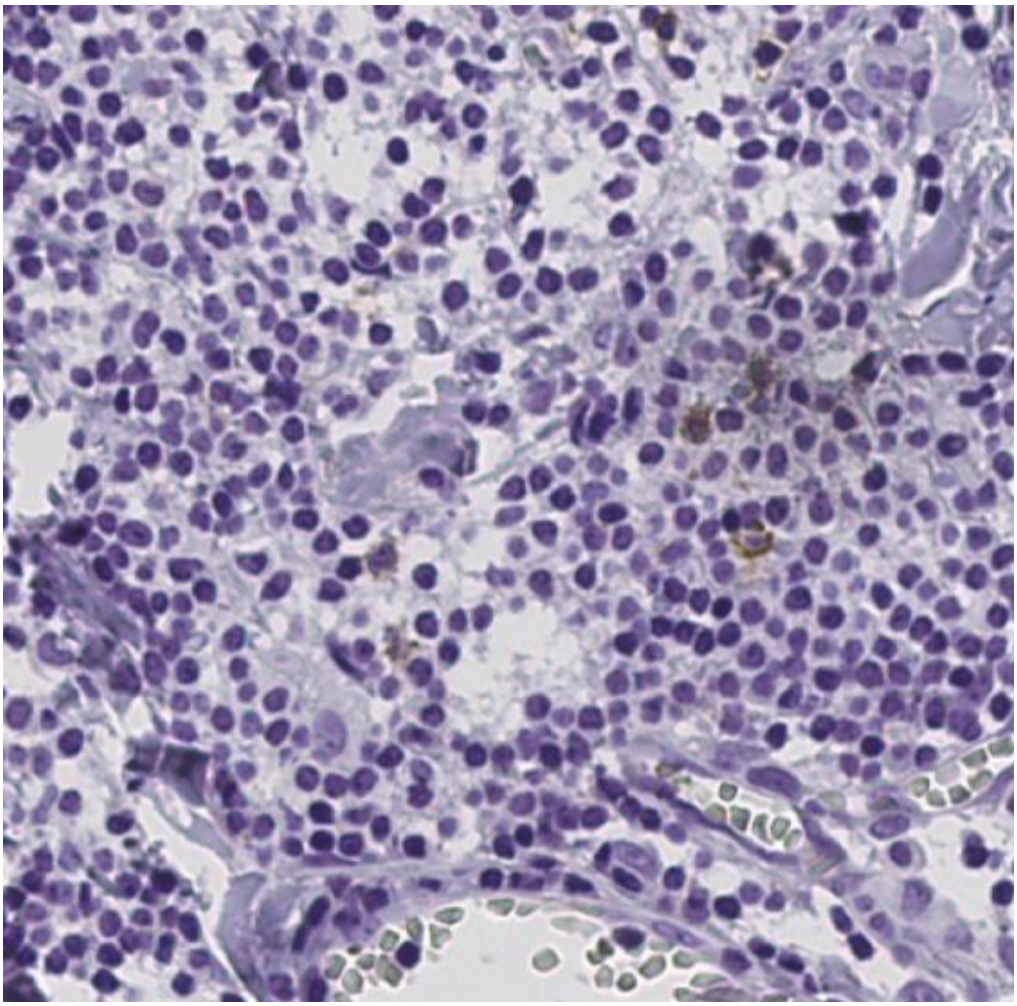
Negative staining



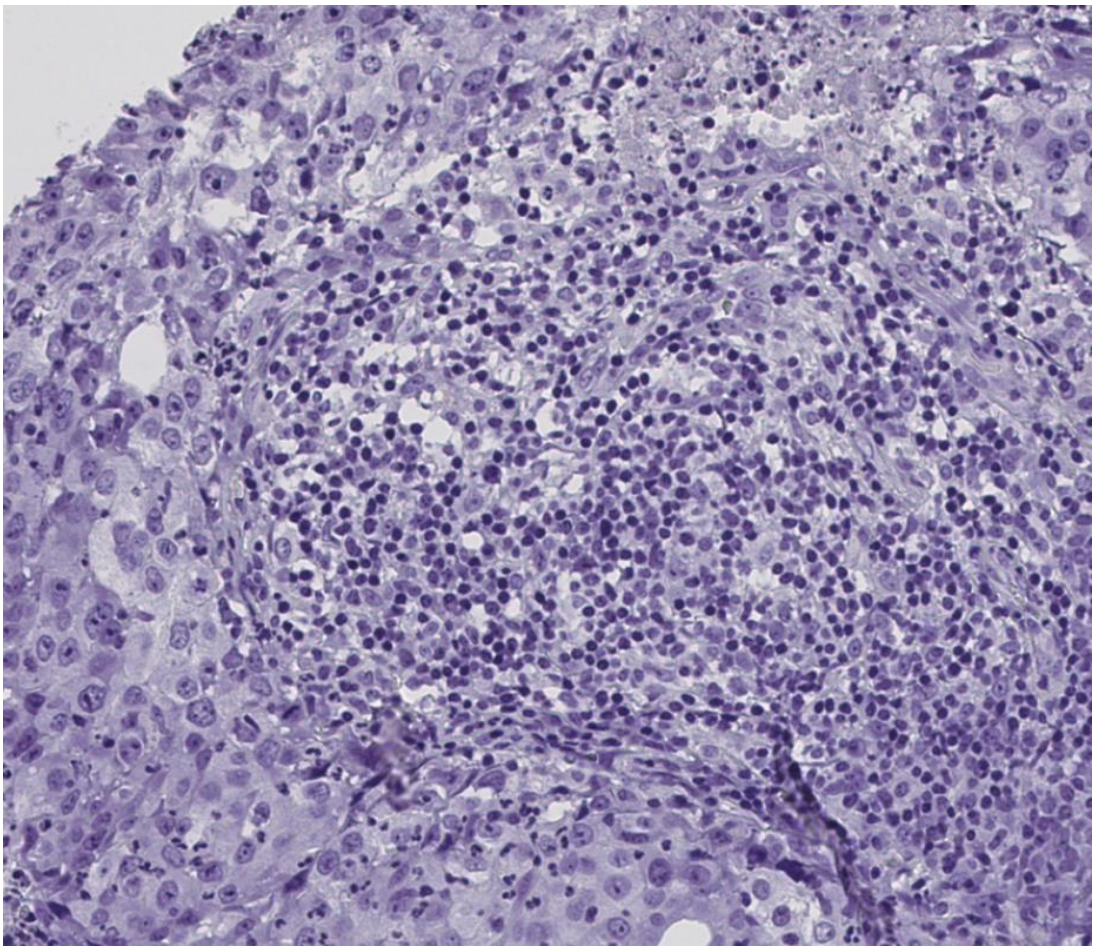
Positive staining (1-9% of lymphocytes)



Positive staining (previous image zoomed)



TILs present but no staining



Protocol immunostaining PD-1

Antibody: Cell Marque 315M-95 (NAT105) in a concentration of 1:50.

Tissue block was cut in 4 micrometer sections and then dried in 60^{o}C for 1 hour.

Deparaffinization and pretreatment was performed in pressure cooker with buffer

pH 6.

The following steps were performed in Autostainer *plus*, DAKO staining equipment with Dako kit K8010 solutions, (except for the primary antibody).

Peroxidase Block	5 min.
Primary antibody	30 min.
EnVision (HRP-conjugated polymers)	30 min.
DAB Substrate-chromogen solution	2x5 min.
Hematoxylin (counterstain)	4 min.
Dehydrate and coverslip	

Between every step, rinse was performed with wash buffer.

Protocol immunostaining PD-L1

Instrument: Benchmark Ultra from Ventana

Antibody: PD-L1 (SP142) (RTU) ref.no 740-4859

Pretreatment buffert: Ultra Cell Conditioning (CC1)/43min in 100 degrees C

Detetion kit: OptiView DAB IHC Detetion kit, ref no 760-700

OptiView Amplification Kit, ref no 860-099

Pretreatment buffert: ULTRA Cell Conditioning 1/CC1) 48min/100 degrees C

Antibody incubation: 16 min/37 degrees C

Background staining: Hematoxylin II/8min, ref no. 740-4859

Using established clinical variables to identify immune responsive tumors in a low-risk cohort

Aims

Immunological biomarkers likely constitute an underutilized source of information in clinical decision making for breast cancer[1]. Despite the large focus on ER-negative subtypes in TILs research, most breast cancers with TILs are ER-positive since the majority of breast tumors are ER-positive[1]. We currently have an incomplete understanding of the role of the immune system in ER-positive breast cancer. We have previously seen that TILs may potentially be used to guide RT individualization in low-risk breast cancer populations. However, to fully be able to utilize this biomarker requires a better understanding of tumor-intrinsic factors affecting immune responsiveness across subtypes rather than within ER-negative subtypes. Furthermore, a better understanding may allow TILs to be used in clinical practice to, in part, guide RT decisions.

We have a unique opportunity of answering the above-mentioned questions in the SweBCG91RT cohort. Our aim is to study if the implications of an immune infiltrate can be predicted by tumor aggressivity, primarily in the form of histological grade, in our low-risk cohort. This may allow for the identification of a subgroup of ER-positive tumors who are immunogenic and may potentially benefit from immunotherapy. Furthermore, we want to investigate if such an understanding may be used to improve RT individualization- an area where additional research is requested by experts within the field[2].

Methods

Study population

The SweBCG91RT cohort. All patients with information on TILs, PD-1, PD-L1, and histological grade will be included. In addition, all patients with high TILs and at least one assessment of PD-1 or PD-L1 of >=1% (even if >=1 TMAs could not be evaluated) as additional TMA evaluations would not change the classification (additional detail for classification under “Analyses”). Among grade II tumors, only those with available gene expression data will be included as gene expression measurements will be used to assess if they will be classified as low- or high-risk (additional details for classification under “Analyses”).

IHC evaluation

Evaluations of PD-1 and PD-L1 from TMAs and TILs from whole sections will be used. Two board-certified pathologists will be evaluating the stainings. The antibodies used for PD-1 and PD-L1, respectively, will be the Cell Marque 315M-95 (NAT105) and Ventana SP142 antibodies. Two cores per

patient and marker are included, and the highest value will be chosen due to the risk of underestimating the degree of positive staining when using TMAs[3]. The same cut-off for positive staining as that in clinical practice will be used (>= 1%) [4].

The reason for including PD-1 and PD-L1 is that they add independent information to TILs which we hypothesize enhances the chances of correctly classifying an immune infiltrate as activated (i.e., having tumor-specific lymphocytes) [1].

Tumor-intrinsic risk group

In a previously unpublished study, we found that a gene expression signature, called Proliferative Index, correlated strongly with histological grade and could be used in a low-risk cohort to predict the biological implications of an immune infiltrate. We, therefore, plan to use histological grade to define immune responsiveness. Grade III tumors will be classified as high-risk and are predicted to benefit from an activated immune infiltrate. Grade I tumors will be classified as low-risk and are not predicted to benefit from an activated immune infiltrate. Grade II tumors constitute a gray zone. They resemble grade I tumors most in terms of Proliferative Index in preliminary assessments and will, therefore, be classified as low-risk unless they have a Proliferative Index above the median of grade III tumors. We believe that classifying the majority of grade II tumors as low-risk in a cohort dominated by ER-positive tumors conforms with the prior literature where an absent or unfavorable prognostic effect from an immune infiltrate is observed[5-7]. The majority of tumors should therefore fall into the low-risk category that does not benefit from an immune infiltrate. Since most tumors are classified as grade II in the SweBCG91RT cohort, this should mean that the majority of grade II tumors would fall into this category. Only grade II tumors with an exceptionally high Proliferative Index (>= median of grade III tumors) will, therefore, be upgraded to high-risk tumors.

Statistics

1. Endpoint: Ipsilateral breast tumor recurrence (IBTR) within 10 years
2. Cox regression analysis to calculate the biologic effect from immune activity and tumor-intrinsic risk group depending on RT in the presence of competing risks
3. Figures: Cumulative incidence functions based on the method described by Fine and Gray with subhazard estimates
4. All analyses are performed in univariable and multivariable analysis including the covariates age, tumor size, ER status, RT
5. Schoenfeld residuals will be used to check proportional hazards assumption

Analyses

6. Classify immune infiltrate into activated or not activated (*immune activity*)
    a. Activated: TILs >= 10% + (PD-1 or PD-L1 >=1%)
    b. Not activated: The remainder of tumors
7. Classify tumors into high- or low-risk (*tumor-intrinsic risk group*)
    a. High-risk: Histological grade III or histological grade II + Proliferative Index >= $\text{median}_{\text{histological grade III}}$
    b. Low-risk: The remainder of tumors (histological grade I or histological grade II + Proliferative Index < $\text{median}_{\text{histological grade III}}$)
8. Analysis of tumor-intrinsic risk group as predictive of immune responsiveness
    a. Interaction test between *tumor-intrinsic risk group* x *immune activity*
9. Analysis of benefit from *immune activity* and *RT* stratified by *tumor-intrinsic risk group*
    a. Cox regression of RT benefit within the four combinations of *immune activity* x *tumor-intrinsic risk group*
    b. Figures cumulative incidence based on Fine and Gray method

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2. Kaidar-Person, O., P. Poortmans, and R. Salgado, *Genomic-adjusted radiation dose to personalise radiotherapy.* Lancet Oncol, 2021. **22**(9): p. 1200-1201.
3. Sobral-Leite, M., et al., *Assessment of PD-L1 expression across breast cancer molecular subtypes, in relation to mutation rate, BRCA1-like status, tumor-infiltrating immune cells and survival.* Oncoimmunology, 2018. **7**(12): p. e1509820.
4. Schmid, P., et al., *Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer.* N Engl J Med, 2018. **379**(22): p. 2108-2121.
5. Sobral-Leite, M., et al., *Cancer-immune interactions in ER-positive breast cancers: PI3K pathway alterations and tumor-infiltrating lymphocytes.* Breast Cancer Res, 2019. **21**(1): p. 90.
6. Liu, S., et al., *Prognostic significance of FOXP3+ tumor-infiltrating lymphocytes in breast cancer depends on estrogen receptor and human epidermal growth factor receptor-2 expression status and concurrent cytotoxic T-cell infiltration.* Breast Cancer Res, 2014. **16**(5): p. 432.
7. Johansson, A., et al., *Clinical and molecular characteristics of estrogen receptor-positive ultralow risk breast cancer tumors identified by the 70-gene signature.* Int J Cancer, 2022.

ONCOLOGICAL CENTER

for the Southern Health Care Region

SB 91: A

Breast conserving treatment with or without postoperative radiotherapy in breast cancer stage T 1-2 (<30 mm), pN0, M0

A national multi-center study

Prepared by

Sydsvenska Breast Cancer Group

1990

Replaces national breast cancer treatment program for Stage I, 1982

PROGRAM DESCRIPTION - STUDY SB 91: A

1. OBJECTIVE

1.1 Primary objective

The study involves a comparison between standardized sector resection and axillary lymph node dissection with or without routine postoperative radiotherapy against residual breast parenchyma in invasive breast cancer stage T 1-2 (max. Diam 30 mm), pN0, M0.

The main aim is to test the hypothesis that local tumor control is achieved without post-operative radiotherapy provided that the surgical procedure is well standardized and aims to optimize the conditions for local radicality.

1.2 Secondary objectives

- Is there a difference in mortality between the two treatment options?

- Is local recurrence mainly due to lack of surgical radicality or due to progression of multicentric changes in other parts of the breast?

- Can patients with high risk of local recurrence already be identified during primary treatment and then selected for post-operative radiation therapy?

- Does local radiation treatment have any effect on the incidence of cancer in the ipsilateral and the contralateral breast?

2. Ethics

2.1 The study has been reviewed and approved by the Research Ethics Committee at Lund University.

2.2 The patient shall be adequately informed of the nature of the disease and current treatment options.

2.3 A template for oral information is attached to the protocol.

2.4 The study has been prepared in accordance with the principles of the Helsinki Declaration 1964.

3. DIAGNOSIS

3.1 Diagnosis of breast cancer is not regulated in this protocol but follows the usual principles. Triple diagnostics (clinical examination, mammography and cytology) should always be performed.

3.2 If required, excision biopsy should, if possible, be carried out in the form of sector resection.

3.3 Biopsy of non-palpable breast changes requires access to the preparation X-ray.

3.4 Tumor tissue tests for steroid receptor determination and DNA analysis should be routinely performed if the tumor size allows such sampling. However, these analyzes are not required for participation in the study.

3.5 In primary surgery, pulmonary X-ray and liver status (S-Car, S-ALP, S-ASAT, S-ALAT, S-GT, S-Ca) are recommended. Further metastatic diagnostics is performed only if clinical or laboratory signs for distant metastasis are present.

4 Patient selection

4.1 Participating clinics should aim to include all patients who fulfill the criteria for randomization. Randomization can occur when all inclusion criteria are met. This means that a definitive result from the histopathological analysis must be present.

4.2 A total of 1100 patients (in the whole country) should be randomized into the study before the inclusion is stopped.

4.3 Patients with tumor stage T1-2 N0 who do not fulfill the eligibility criteria according to 4.4 should be treated according to the current guidelines.

4.4 Eligibility criteria

4.4.1 Female with invasive primary breast cancer.

4.4.2 The patient's age should not exceed 75 years on the day of the operation. No lower age limit.

4.4.3 No signs of distant metastases during preoperative complete clinical examination.

4.4.4 If the tumor is radiologically visible, preoperative mammography should show a tumor diameter of maximum 30 mm. The mammography must not show multiple tumors in multiple quadrants and not microcalcifications beyond the sector which can be safely excised with the tumor.

4.4.5 The tumor should be excised with a sector resection as primary surgery procedure or as a resection after diagnostic biopsy.

4.4.6 Radical excision according to histopathological assessment. If the radicality is uncertain, a completing radical excision is necessary.

4.4.7 Histopathological analysis should show tumor radicality and abscence of signs of multifocal cancer which includes invasive cancer or cancer in situ further than 2 cm away from the periphery of the primary tumor.

4.4.8 No signs of lymph node metastases in histopathological analysis after axillary lymph node dissection. At least 5 lymph nodes should be analyzed.

4.4.9 The relationship between tumor and breast size should allow local radicality with an acceptable cosmetic result.

4.4.10 The patient is informed and accepts sector resection with or without radiotherapy as definitive treatment.

4.4.11 It is possible to follow the patient and the patient should not suffer from another serious illness, such as severe dementia, a severe psychological disorder or drug addiction problems.

4.4.12 The patient can participate in the radiotherapy treatment.

5. Randomization

5.1 Patients who meet all of the eligibility criteria according to 4.4.

5.2 Randomization is done by phone 046 - 17 75 60 to Oncologic Center, Lasarettet, Lund.

5.3 Randomization occurs directly after the results of the histopathological examination are definitive.

5.4 Stratification takes place for participating clinics and if the tumor is detected by screening or not.

5.5 Randomization occurs to either of the two treatment options:

- Postoperative radiotherapy against the operated breast.

- No postoperative radiotherapy.

5.6 Randomization occurs simultaneously for adjuvant treatment in accordance with study protocol SB 91: B

5.7 Patients randomized to radiotherapy are immediately referred to oncologic clinic with information that the patient participates in the study.

5.8 Breast cancer application form is submitted immediately after randomization to the Department of Oncology Center.

6. POST-OPERATIVE TREATMENT

6.1 Radiotherapy

Postoperative radiotherapy is initiated as soon as the wound has healed and is administered according to the technical description in the national guidelines.

6.2 Adjuvant treatment

Premenopausal patients should receive adjuvant treatment according to SB 91: B (separate study). Postmenopausal women with tumor size 21-30 mm should also be included in SB II: 2 and be randomized to Tamoxifen for 2 and 5 years respectively.

6.3 Treatment of local recurrence

Treatment of local recurrences in remaining breast parenchyma is not regulated in this protocol and should be determined in a joint consultation between the patient and the treating physician. In the group treated with radiotherapy, mastectomy is recommended. In limited recurrences in the breast among patients not treated with radiotherapy, a new local excision with adjuvant radiotherapy should be considered.

6.4 Treatment of regional recurrences and distal metastases

This treatment is not regulated in this protocol.

7. FOLLOW-UP

7.1 All patients are followed by clinical examination every six months for two years, then annually to 5 years.

7.2 Clinical mammography is performed annually for 10 years.

7.3 After 10 years, patients are referred to general health examination with mammography.

7.4 Results after 10 years are requested.

7.5 All randomized patients should be checked in the above manner regardless of whether they completed treatment or not.

7.6 In addition, additional controls may be considered, for example, in case patients are included in another study.

7.7 Results of each control are reported on the form "Follow-up of breast cancer patients".

8. DEFINITION OF CLINICAL EVENTS

8.1 Local recurrence

Registration of recurrence in the treated breast constitutes the primary purpose of the study.

All cytologically or histopathologically verified relapses in remaining breast parenchyma or in cutis and subcutis adjacent to the breast are classified as local relapse.

When local recurrence occurs, they should, if possible, be classified into either of the following categories:

- recurrence in previously operated areas
- recurrence / new tumor in the breast parenchyma outside of the previously operated area
- recurrence in intramammaric lymph node or as emboli in lymph vessels
- recurrence outside of the breast parenchyma, i.e. in cutis or subcutis

Subsequently diagnosed lymph node metastases, for example processus axillaris, are classified as regional recurrence.

If there is difficulty in clearly referring a patient to one of the four above-mentioned groups, the case is referred to the project group for classification without the knowledge of the treatment group.

8.2 Death

In the event of death, autopsy is sought. The pathologic departments should be advised to pay special attention to whether they receive autopsy for breast cancer surgery on the basis of breast cancer.

When patients randomized to the study die, one should seek classification in one of the following categories:

- death due to breast cancer

- death with residual breast cancer but with other major cause of death.

- death without signs of breast cancer relapse

If there is difficulty in clearly referring a patient to one of these categories, the case is referred to the project group for classification without the knowledge of the treatment group.

9 EVALUATION AND STATISTICS

9.1 The endpoints for the analysis are local recurrence, new primary tumor or death. Analysis of relapsed survival and total survival should be performed for all randomized patients.

9.2 Material Size

To determine the overall size of the study, it is assumed that the expected local recurrence rate after 5 years is 5% in the radiotherapy group. Below is the required total size of the study for one or two-sided tests on the significance level 0.05 with the probability 0.80 to detect expected local recurrence rates in the non-irradiated group of 7%, 9%, 10% and 11%.

Expected Share Required mtrl size (total)

local recurrence (alpha = 0.05, beta = 0.80)

+ RT	-RT	
	Two-sided test	One-sided test
0,05	0,07	3500
	4400	
0,05	0,09	1000
	1280	
0,05	0,10	680
	870	

0,05	0,11 640	500

When it can be ruled out in advance that the local rate of return is lower in the non-radiotherapy group, the study should be dimensioned based on a one-sided test. With 1000 patients, one can then detect a difference in the expected frequency from 0.05 to 0.09 with the probability of 0.8. With correction for 5% loss, the material size will then reach 1100 patients.

9.3 Interim Assessment

Interim evaluation should be done when 600 patients are randomized. The study should be discontinued if there is a significantly higher mortality ($p < 0.05$) in either group or if a life-table estimate of local recurrence rate after three years is above 15% for the non-radiated group.

Cancellation decisions take place after the meeting with the project team.

9.4 Management alignment

This study is management-oriented, which means that patients are analyzed in the group to which they have been randomized, regardless of whether treatment is completed or not.

9.5 Analysis

The statistical processing will be done with unidentified treatment groups.

10. ADMINISTRATION

10.1 The study is planned in consultation with the National Association against Cancer Planning Group for Breast Cancer. This group, as well as the work committee for the study, constitutes a national management team which is responsible for long-term continuity within the study and initiates the scientific evaluations. The management team coordinates locally initiated sub-projects.

10.2 The work committee for the study consists of Lars Holmberg, Uppsala; Stefan Rydén, Ängelholm; Lars-Erik Rutqvist, Stockholm; John Carstensson, Linköping and by a contact person for each participating region.

The work committee is responsible for the practical management of the study and is responsible for the management team. The work committee manages ongoing organizational

and scientific issues within the study, monitoring data quality and conducting secretarial functions.

10.3 The study forum for information, discussion and coordination is a project group. This consists of a responsible person from each participating clinic. The management team is represented in the project committee of the work committee. The project group discusses all fundamentally important questions in the study.

10.4 Randomization and dispatch and collection of forms and data readings take place at the Oncological Center, Lasaret, Lund, which is responsible for validation and monitoring. A secretary directly under the work committee is responsible for contacts between the work committee and the regional oncology centers, recurrent compilations of fact quality control and reports.

11. PUBLICATIONS

11.1 Each publication based on the clinical material is based on all participating clinics and oncological centers. The presentation will take place throughout the project group and management team name. To each publication, an addendum is attached clearly indicating who have been actively involved in the study's implementation at the various clinics, processing and completion of the script.

11.2 The co-authorship of publications analyzing special aspects such as histopathology, mammography, DNA content or hormone receptor content- will be discussed jointly between the participants in the project. In principle, the Medical Association's guidelines must be followed (Läkartidningen 79: 2454-2455, 1982).

11.3 Each clinic participating in the study may use the material for regional or local information in the form of lectures. However, it should always be stated which clinics participated in the survey.

11.4 Local-initiated sub-projects within the study are published by the respective project managers.

TEMPLATE FOR PUBLIC PATIENT INFORMATION

Background

This study is based on the belief that mortality will not differ between the radiation treated and the non-radiated group. On the other hand, the incidence of undiscovered multicentric changes may cause a higher local rate of recurrence in the non-radiated group. The local recidivism is most likely to be detected early in the annual mammography controls. Renewed local excision followed by radiation therapy against residual breast parenchyma should therefore be possible in most cases. In view of this, the disadvantages of routine postoperative radiation treatment may be greater than the benefits.

Discussing these considerations in detail with the individual patient would be difficult and often contrary to the Helsinki Declaration's position that information should not be given if it injures the patient.

Previous experience of oncological trials showed that it is difficult properly to inform patients about the randomization process and to putt hem at an open choice in the form of two treatment options without support or recommendation from treating physicians.

The patient should be informed that a study is in progress according to the following guidelines and that the patient will be included in the study if they accept the treatment of the doctor. The information should of course be adapted to any participating clinic according to suggestions and requirements from local or regional ethics committee.

Information

You are, as we now know, in a favorable disease state with limited tumor without spreading to the lymph nodes in the armpit. Therefore, we do not consider the treatment to be improved if the entire breast is removed but instead propose that only the part of the breast containing tumor is removed.

After the surgery there are two different treatment options. One means that radiation treatment is given to the breast for about 5 weeks. On the other hand, radiation therapy is only provided

if you develop tumor recurrence in the breast. You must first do a renewed surgery, but in the number of cases your chest can be preserved.

Nothing suggests that the different finishes differ in terms of the most important outcome: the chance of lasting cure. The difference is in local effects. After radiation therapy, there is a certain risk of radiation effects in the short and long term. In surgery without radiation treatment there is a slightly increased risk of local relapses.

Which postoperative treatment is best is an undisclosed question. We therefore propose that we determine what treatment should be given as part of an investigation.

This is then determined according to a predetermined list. You are in full right to refuse participation or to cancel participation in the survey and may then choose the treatment you wish. Parts of the journal will be processed without the possibility of identifying that participation.