ACTA ONCOLOGICA
2022, VOL. 61, NO. 2, 153–162
https://doi.org/10.1080/0284186X.2021.1989629

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Tumor-infiltrating lymphocytes predict improved overall survival after post-mastectomy radiotherapy: a study of the randomized DBCG82bc cohort

Trine Tramm[a], Hanne Vinter[a], Pernille Vahl[a], Demet Özcan[a], Jan Alsner[b] and Jens Overgaard[b]

[a]Department of Pathology, Aarhus University Hospital, Aarhus, Denmark; [b]Department of Experimental Clinical Oncology, Aarhus University Hospital, Aarhus, Denmark

ABSTRACT

Background: The predictive value of tumor-infiltrating lymphocytes (TILs) on the benefit from radiotherapy (RT) remains unclear. Our aim was to investigate the association between TILs and post-mastectomy RT (PMRT) regarding the risk of recurrence and survival in a randomized cohort.

Material and methods: Stromal TILs were histologically estimated in 1011 tumors from high-risk breast cancer (BC) patients from the DBCG82bc trial. Patients were diagnosed between 1982 and 90, treated with total mastectomy and partial axillary lymph node dissection, randomized to ± PMRT followed by adjuvant systemic treatment. A competing risk model, Kaplan–Meier analysis and multivariate Cox regression analysis were used for correlating TILs and clinical outcome.

Results: 106 of 1011 patients (10.5%) showed high TILs using a 30% cut-off. In multivariate regression analysis, a high level of TILs was an independent factor associated with lower risk of distant metastasis (DM) and improved overall survival (OS), but without association with loco-regional control. High TILs were associated with a significantly greater OS after PMRT at 20 years compared to low TILs (8% improvement for low TILs (23% to 31%) vs. 22% for high TILs (26% to 48%), interaction-test: $p = 0.028$). The association between TILs and PMRT was more pronounced among estrogen-receptor negative (ER–) tumors, and patients having ER–/low TILs tumors showed no OS benefit from PMRT at 20 years (–4% improvement for low TILs (28% to 24%) vs. 23% for high TILs (20% to 43%). A similar trend in the association between high TILs and reduced risk of DM after PMRT was seen.

Conclusion: High TILs predict improved OS from PMRT in BC patients, and the association appeared especially strong for ER– tumors. A trend in the association between high TILs and reduced risk of DM after PMRT was seen. These findings may indicate that RT triggers an immune response inducing a systemic effect outside the treatment field.

ARTICLE HISTORY
Received 27 June 2021
Accepted 1 October 2021

KEYWORDS
Breast cancer; radiotherapy; tumor-infiltrating lymphocytes; mastectomy; survival

Introduction

Tumor-infiltrating lymphocytes (TILs) represent a surrogate marker for the adaptive immune system. Around 10–11% of breast cancers (BC) have been found to contain a high number of TILs, and these are usually more aggressive and highly proliferative, estrogen receptor (ER) negative tumors with either a triple-negative (TN) or HER2 positive (HER2+) phenotype. Both ER-positive (ER+) (luminal) and ER-negative (ER–) (non-luminal) cancers can, however, show low, intermediate and high levels of TILs [1].

A high level of TILs has been associated with improved recurrence-free- and overall survival (OS) especially in patients with TN and HER2+ breast cancers [2–4]. An adverse prognostic effect of high TILs has on the other hand been described in patients with ER+/HER2 negative (HER2–), luminal tumors, which is suggested to be related to immunomodulating effects of anti-estrogen treatment [5]. Furthermore, a high level of TILs is more predictive of response to neoadjuvant chemotherapy in all subtypes of breast cancer [5]. TILs have also been investigated as a predictive marker of response to PD-1/PD-L1 inhibitors in BC patients, and it has been proposed to add evaluation of TILs to PD-L1 expression to strengthen the predictive value of the latter [6].

The association between TILs and radiotherapy (RT) is less well-described, and the effect of RT has complex and opposing effects on the immune system including upregulation of immune checkpoints (e.g., PD-L1) contributing to a generalized immunosuppressive microenvironment [7,8]. The association has been examined in the SweBCG91RT trial of BC patients treated with breast-conserving surgery (BCS) and randomized to ±RT [9,10]. The findings indicated a positive association between high TILs and reduced risk of ipsilateral breast tumor recurrence (IBTR), suggesting that RT would be more beneficial in patients with low TILs [9]. Currently, it is investigated in which way the delivery of RT may affect the immune response [11–13]. This includes investigation of

CONTACT Trine Tramm tramm@oncology.au.dk Department of Pathology, Aarhus University Hospital, Palle Juul-Jensens Boulevard 99, plan 1, C112, Aarhus N, DK-8200, Denmark

Supplemental data for this article can be accessed here.

possible systemic effects of RT induced by local immune-priming effects resulting in the circulation of effector immune cells to distant sites, as well as how the immune response might be shaped and systemic effects induced by specific RT regimes (dose and fractionation) and combinations of RT with chemo- and/or immunotherapy [13–16].

Loco-regional RT is aimed at eliminating tumor stem cells that may remain in the loco-regional area after surgery. After mastectomy, this will primarily encompass eradication of residual tumor cells located in the chest wall and regional lymph nodes. If left untreated, these residues may lead to local or distant recurrences (or a local recurrence may act as a nidus for distant recurrence) and eventually death from BC. Not all BC patients gain benefit from RT and some patients may only suffer the risk of RT associated early and late side-effects, including ischemic heart disease [17,18] and secondary cancer [19]. The effect of RT on survival has further been shown to vary with tumor biology (e.g., ER, HER status), as aggressive tumors with a predilection of early metastatic seeding (e.g., TN and HER2+ BC) are less likely to achieve a survival benefit by RT through local control [20]. A previous study of the DBCG82bc cohort (Danish Breast Cancer Group) showed a reduction of loco-regional recurrences (LRR) after PMRT among all BC subtypes, translating only into improved overall survival (OS) in patients with ER+ and/or HER2– tumor [20]. On the other hand, ER+/HER2– tumors, especially with a Luminal A subtype, are associated with a lower risk of LRR than the other subtypes [21–23], and ongoing clinical trials, e.g., the DBCG Natural trial (*ClinicalTrials.gov, NCT03646955)* and the EXPERT trial (*NCT02889874)* are investigating the effect of omitting RT after BCS in selected patients with ER+/HER2– tumors.

Given the varying benefit of RT among BC patients, the risk of acute and late effects of RT and the decreasing LRR rate [24–27], it is of utmost importance to refine the identification of patients with more or less benefit from RT and focus on the indirect systemic effects on distant metastasis (DM).

We have previously developed and validated a predictive gene-expression profile (DBCG-RT profile) capable of identifying patients with benefit from PMRT in the DBCG82bc cohort [28]. The genes in the profile correlated with various parts of the immune system, *e.g.,* inflammatory response, T-cell activation and differentiation and antigen-presenting.

Now, we aim to investigate the predictive value of TILs in terms of benefit from PMRT in pretreatment tumor material from the DBCG82bc cohort of high-risk BC patients randomized to RT or not and to examine if a possible association are dependent on the ER status.

Material and methods

Patients

The DBCG82bc cohort has been described in detail elsewhere [29–32]. In brief, 3083 BC patients treated with mastectomy and partial axillary dissection and diagnosed in the period 1982–1990 were included. All patients received systemic therapy according to menopausal status. Premenopausal women were treated with cyclophosphamide, methotrexate and fluorouracil (CMF), and postmenopausal women with tamoxifen. All patients were under the age of 70 and considered to be at high risk of recurrence due to positive lymph nodes and/or tumor size >5 cm and/or invasion in the skin or pectoral fascia. A median of 7 axillary lymph nodes was removed.

From a total of 1011/3083 patients, tumor-containing paraffin blocks collected for previous studies of the DBCG82bc cohort [20,28] were available and contained sufficient invasive carcinoma to be included in the current study of TILs, as depicted in the CONSORT diagram (Supplemental Figure 1). A total of 932 pts. (92%) had more than 7 lymph nodes removed.

ER– and Progesterone (PR) receptor–, HER2–receptor status and Ki-67 index were available from immunohistochemical analysis (IHC) analyses on Tissue Micro Arrays (TMA) carried out as part of the previous studies [20,28]. For ER and PR, a cut-off of 1% was used (≥1%). HER2 evaluation followed the ASCO 2007 guidelines [33] applicable at the time of analysis. For two patients, HER2 status was not available. A cut-off of 20% was used for separating high (≥ 20%) and low Ki-67 (<20%).

The evaluation of TILs was performed on Hematoxylin-Eosin stained, whole slides. The evaluation followed the recommendations from the TILs working group 2014 [34] and was carried out by two observers independently (TT, HV). In short, TILs encompass both lymphocytes and plasma cells, and only stromal TILs within the borders of the invasive tumor were evaluated. The estimation was semiquantitative, assessing an average TILs score in the tumor area for the whole section with no evaluation of hotspots. Each TILs value describes the 'area of stromal tissue occupied by TILs/total area of intra-tumoral stromal area'. TILs were recorded as a continuous variable, and hereafter, the amount of TILs were categorized into various 2- or 3-tier categorizations using different cut-offs (0–10%, 11–39%, ≥40%; 0–20%, 21–49%, ≥50%; <10% vs. ≥10%; <20% vs. ≥20%; <30% vs. ≥30%; <40% vs. ≥40%; <50% vs. ≥ 50%; <60% vs. ≥60%).

The interobserver agreement between the two raters in assessing TILs showed an intraclass correlation coefficient (ICC) including a 95% confidence interval (CI) above 0.70 and an overall concordance for all categories in the range of 0.84–0.97 (Supplemental Table 1). A cut-off value of 30% for distinguishing between low and high TILs was chosen for subsequent analyses since this cut-off was associated with the best combination of high Fleiss kappa value and an optimal concordance rate. This led to 106 patients (10.5%) being categorized as having tumors with high TILs levels and 905 (89.5%) with low TILs levels (Figure 1).

The study was approved by The Central Denmark Region Committees on Health Research Ethics (journal no. 1-10-72-45-17), The Danish Data Protection Agency (journal no. 1-16-02-698-17), and reporting follows Recommendation for Tumor Marker Prognostic Studies (REMARK) criteria [35].

Statistical analysis

Inter-observer agreement for the evaluation of TILs reported as a continuous score was assessed *via* the ICC calculated

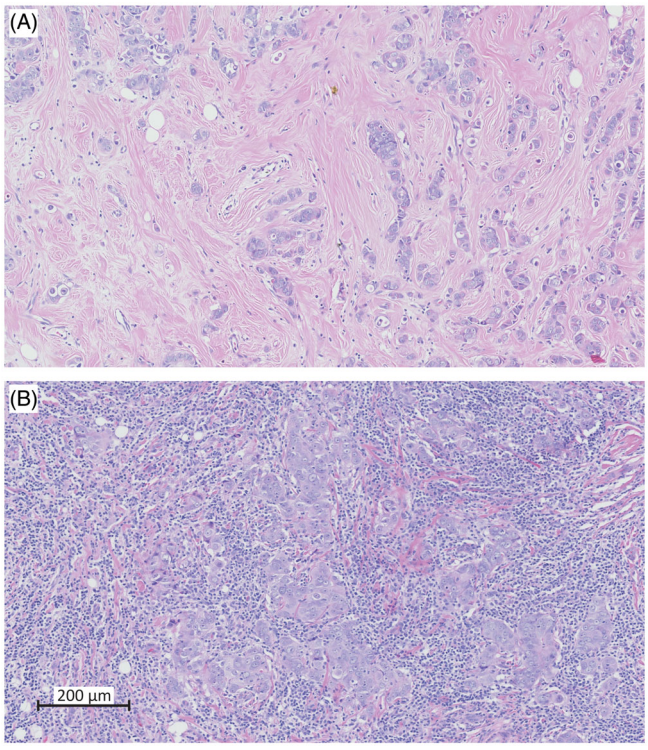

Figure 1. Hematoxylin–eosin (HE) staining's showing examples of tumors with (A) low level of stromal tumor-infiltrating lymphocytes (TILs) and (B) high level of TILs using a cut off of 30%.

using a mixed model. No standard values for an acceptable agreement for ICC exist, but an ICC of >0.70 (including 95% CI > 0.70) has previously been used as an endpoint for a successful evaluation of TILs [36,37]. Fleiss kappa (for 2 raters) was used for assessing the inter-observer agreement, when evaluating TILs as categorical data [38,39], as well as concordance rates (=percentage of agreement between raters in predicting a low, (intermediate) or high level of TILs).

A competing risk model, Kaplan–Meier analysis and multivariate Cox regression analysis (MVA) were used for analyzing correlations between TILs and clinical outcome. The endpoints considered were LRR, DM and OS. LRR was defined as the appearance of ipsilateral chest wall failure (local) or ipsilateral axillary or supra/infraclavicular failure (regional). DM was defined as any failure outside the ipsilateral mammary region and the regional lymph nodes. The following events were competing with LRR: DM >1 month before LRR, contralateral breast cancer (CL) >1 month before LRR/DM, second primary cancer (SPC) >1 month before LRR/DM/CL, and death without any other event. The following events were competing with DM: CL >1 month before DM, SPC >1 month before DM/CL, and death. Patients alive with no events were censored at the last date of vital status/follow-up. Effects of competing risks were evaluated by fitting cumulative incidence functions, and cumulative probability curves were calculated and tested for differences. Survival outcome was calculated from the date of surgery. Cox uni- and multivariate regression analyses were performed, and assumptions of proportional hazards were tested graphically using log-minus-log plots. The MVA was stratified on ER status which did not show proportional hazards. Interaction tests were calculated on adjusted hazard ratios (HR) from the MVA (adj. interaction test) as well as on risk differences. The level of significance was 5% and all estimated p-values were two-sided. Statistical analysis was performed using STATA-version 16.1 (StataCorp, College Station, TX).

Results

Table 1 shows the demographic, clinical and pathological characteristics of data for the patients included in the present study. Patients were equally distributed between the randomization arms (±PMRT). The level of TILs was significantly associated with malignancy grade, histology type and ER– and HER2 status, but not with age, menopausal status (protocol), tumor size or nodal status. For 1009 patients (99.8%), IHC defined subtypes could be constructed from ER–, PR–, HER2 status and Ki-67. Tumors with high as well as low TILs were found among all subtypes, though the distribution was statistically significantly different (Fischer exact test, $p < 0.0001$). As expected, high TILs were more frequently seen among non-luminal tumors (ER- and PR- tumors), whereas the majority of ER+/HER2– cancers showed low levels of TILs. High TILs were further associated with having a 'high LRR risk' score according to the DBCG-RT profile.

After a median follow-up time of 25 years, a total of 232 LRR and 580 DM were observed, corresponding to cumulative incidences of 23% and 58% for risk of LRR and DM, respectively. In the low TILs group, cumulative incidences were 22% (200/905) and 58% (525/905) for risk of developing LRR or DM, whilst cumulative incidences in the high TILs group were 30% (32/106) and 51% (55/106) for LRR and DM, respectively.

In a univariate analysis encompassing all 1011 patients, TILs level was not associated with either risk of LRR, DM or with OS (Table 2). However in an MVA, high TILs were associated with a significantly lower risk of DM (adj. HR= 0.62, CI: 0.46–0.83) and improved OS (adj. HR = 0.65, CI: 0.50–0.84), when adjusting for PMRT, menopausal status, T- and N-stage, histology type, malignancy grade and HER2 status and stratified by ER. An association between TILs and LRR was not found in MVA (adj. HR= 1.00, CI: 0.67–1.50).

The MVA was repeated using 20% and 40% as cut-off values for low vs. high TILs. This led to similar results as for a 30% cut off with non-significant associations between TILs and LRR (20%; HR = 0.97 (0.67–1.39, $p = 0.85$ and 40%; HR-0.91 (0.59–1.42), $p = 0.69$), but significant associations with DM (20%; HR = 0.60 (0.47–0.78), $p < 0.0001$ and 40%; HR-0.65 (0.48–0.90), $p = 0.0087$) and OS (20%; HR = 0.70 (0.57–0.87), $p = 0.0014$ and 40%; HR-0.68 (0.51–0.90), $p = 0.0065$).

A significant positive association between high TILs and OS was observed after PMRT, illustrating the predictive value of TILs in terms of benefit from PMRT. For all clinical endpoints, a benefit from PMRT could be seen regardless of TILs level (Figure 2(A–F)). The reduced risk of LRR after PMRT was of similar size for patients with low and high TILs (24% *vs.* 26% difference at 20 years, Figure 2(A,B)). A trend for greater

Table 1. Patient characteristics.

	All	Low TILs (<30%)		High TILs (≥30%)		
	(*n* = 1011) *n*	No PMRT (*n* = 458) *n* (%)	PMRT (*n* = 447) *n* (%)	No PMRT (*n* = 47) *n* (%)	PMRT (*n* = 59) *n* (%)	*p*-value*
Age (years)						
Median (range)	53 (25–69)	52 (25–69)	53 (30–69)	53 (31–68)	53 (27–67)	
Age (number)						
<50 years years	409	203 (44%)	161 (36%)	21 (45%)	24 (41%)	.68
≥50 years years	602	255 (56%)	286 (64%)	26 (55%)	35 (59%)	
Menopausal status/protocol						
Premenopausal/CMF	553	258 (56%)	236 (53%)	26 (55%)	33 (56%)	.92
Postmenopausal/Tam	458	200 (44%)	211 (47%)	21 (45%)	26 (44%)	
Tumor size						
≤20mm	362	166 (36%)	160 (36%)	17 (36%)	19 (32%)	.64
21–50mm	506	216 (47%)	238 (53%)	22 (47%)	30 (51%)	
>50mm	143	76 (17%)	49 (11%)	8 (17%)	10 (17%)	
Positive nodes						
0	59	28 (6%)	24 (5%)	3 (6%)	4 (7%)	.73
1–3	462	199 (43%)	218 (49%)	21 (45%)	24 (41%)	
≥4	490	231 (50%)	205 (46%)	23 (49%)	31 (53%)	
Malignancy grade						
Grade 1	231	111 (24%)	113 (25%)	3 (6%)	4 (7%)	<.0001
Grade 2	538	260 (57%)	240 (54%)	18 (38%)	20 (34%)	
Grade 3	234	83 (18%)	91 (20%)	26 (55%)	34 (58%)	
Not graded	8	4 (1%)	3 (1%)	0 (0%)	1 (2%)	
Histology type						
Ductal carcinoma	898	386 (84%)	410 (92%)	46 (98%)	56 (95%)	<.0001
Lobular carcinoma	92	61 (13%)	31 (7%)	0 (0%)	0 (0%)	
Other carcinomas	21	11 (2%)	6 (1%)	1 (2%)	3 (5%)	
ER status						
Negative < 1%	274	101 (22%)	106 (24%)	30 (64%)	37 (63%)	<.0001
Positive ≥ 1%	737	357 (78%)	341 (76%)	17 (36%)	22 (37%)	
HER2 status						
Negative	787	371 (81%)	356 (80%)	26 (55%)	34 (58%)	<.0001
Positive	222	86 (19%)	90 (20%)	21 (45%)	25 (42%)	
Unknown	2	1 (0%)	1 (0%)	0 (0%)	0 (0%)	
Subtypes						
ER + or PR+[a]/HER2–/Ki67 low[b] or unknown	557	280 (61%)	258 (58%)	10 (21%)	9 (15%)	<.0001
ER + or PR+/HER2–/Ki67 high[b]	106	44 (10%)	53 (12%)	4 (9%)	5 (8%)	
ER + or PR+/HER2+/Any Ki67	119	50 (11%)	51 (11%)	6 (13%)	12 (20%)	
ER– and PR–/HER2+/Any Ki67	103	36 (8%)	39 (9%)	15 (32%)	13 (22%)	
ER– and PR–/HER2–/Any Ki67	124	47 (10%)	45 (10%)	12 (26%)	20 (34%)	
Unknown	2	1 (0%)	1 (0%)	0 (0%)	0 (0%)	
DBCG RT-profile[c]						
High LRR risk	190	80 (72%)	88 (78%)	11 (100%)	11 (92%)	.034
Low LRR risk	57	31 (28%)	25 (22%)	0 (0%)	1 (8%)	

*p-value is from Fisher's exact test for distribution among low TILs vs. high TILs (irrespective of treatment)).
[a]Cut-off value for estrogen receptor (ER), progesterone receptor (PR): negative <1%, positive ≥1%.
[b]Cut-off value for Ki67: low <20%, high ≥20%.
[c]Data from 247 patients included in the formalin-fixed, paraffin-embedded cohorts in Tramm et al. [28].
Abbreviations: CMF: cyclophosphamide, methotrexate and fluorouracil; Tam: tamoxifen.

reduction in risk of DM was seen (10% reduction for low TILs (63% to 53%) *vs*. 19% for high TILs (62% to 43%) at 20 years), but the adjusted interaction test was not significant ($p = 0.28$) (Figure 2(C,D)). A significantly greater OS benefit at 20 years after PMRT was, however, observed for patients with high TILs as compared to patients with low TILs in the tumor (8% improvement for low TILs (23% to 31%) vs. 22% for high TILs (26% to 48%)) with a significant adjusted interaction test ($p = 0.028$) (Figure 2(E,F)). The interaction test was only statistically significant with the chosen cut-off value of 30% and not using a 20% or 40% cut-off value.

The association between TILs and PMRT appeared stronger among patients with ER- tumors. When stratifying the patients according to ER status, only patients with ER–/high TILs tumors showed benefit from PMRT in terms of OS, whereas patients with ER–/low TILs (207/1011) BC was seen to derive no benefit from PMRT (–4% improvement for low TILs (28% to 24%) vs. 23% for high TILs (20% to 43%)) (Figure 3 (A,B)). The adjusted interaction test was non-significant ($p = 0.092$), but the difference in risk difference was 27% among ER– patients (interaction test (risk difference), $p = 0.029$) compared to 8% among ER + patients (Figure 3 (C,D)) and 14% in the total cohort (Figure 2(F)). The stratification according to ER status led to a limited number of patients in each group (except the ER+/low TILs group), and the 39 patients in the ER+/high TILs group were too few for obtaining reliable results. The risk reduction after PMRT in terms of OS was, nevertheless, similar to that of the 67 patients with ER-/high TILs BC (23% *vs*. 19% difference in ER– and ER+, respectively) (Figure 3(B–D)). Supplemental Figure 2 further shows how the ER–/low TILs group differs from the remaining patients (either high TILs or ER + tumors).

The association between high TILs and risk of DM after PMRT also appeared strongest among patients with

Table 2. Uni- and multivariate Cox regression.

	All			
	Univariate		Multivariate*	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Loco-regional recurrence				
PMRT				
PMRT vs. no PMRT	0.26 (0.19,0.35)	<.00001	0.23 (0.17,0.31)	<.00001
Menopausal status/protocol[a]				
Post- vs. premenopausal	1.00 (0.77,1.30)	1.00	1.06 (0.81,1.39)	.67
T-size				
21–50mm vs. ≤20mm	1.52 (1.13,2.06)	.0058	1.41 (1.04,1.92)	.027
≥51mm vs. ≤20mm	3.04 (2.09,4.43)	<.00001	2.10 (1.42,3.11)	<.001
Positive nodes				
1–3 vs. none	2.08 (0.84,5.14)	.11	2.63 (1.05,6.56)	.038
≥4 vs. none	6.38 (2.61,15.56)	<.0001	7.36 (3.00,18.05)	<.0001
Malignancy grade†				
Grade 2 vs. grade 1	1.40 (0.98,1.98)	.064	1.17 (0.82,1.68)	.38
Grade 3 vs. grade 1	2.43 (1.66,3.56)	<.00001	1.76 (1.14,2.72)	.011
Histology type				
Lobular carcinoma vs. ductal	0.98 (0.63,1.54)	.93	0.79 (0.49,1.28)	.34
Other carcinoma vs. ductal	0.87 (0.36,2.12)	.76	0.83 (0.26,2.61)	.74
HER2				
Positive vs. negative	1.62 (1.20,2.18)	.0014	1.17 (0.85,1.61)	.35
TILs				
≥30% vs. <30%	1.38 (0.95,2.01)	.090	1.00 (0.67,1.50)	1.00
Distant metastasis				
PMRT				
PMRT vs. no PMRT	0.73 (0.62,0.86)	<.001	0.73 (0.62,0.86)	<.001
Menopausal status/protocol[a]				
Post- vs. premenopausal	1.07 (0.91,1.26)	.40	1.08 (0.92,1.28)	.34
T-size				
21–50mm vs. ≤20mm	1.45 (1.21,1.75)	<.0001	1.23 (1.02,1.48)	.034
≥51mm vs. ≤20mm	2.47 (1.93,3.16)	<.00001	1.71 (1.33,2.21)	<.0001
Positive nodes				
1–3 vs. none	1.06 (0.69,1.64)	.79	1.20 (0.77,1.87)	.42
≥4 vs. none	3.49 (2.29,5.32)	<.00001	3.50 (2.28,5.36)	<.00001
Malignancy grade[b]				
Grade 2 vs. grade 1	1.41 (1.14,1.76)	.0019	1.20 (0.96,1.50)	.11
Grade 3 vs. grade 1	2.15 (1.68,2.74)	<.00001	1.74 (1.33,2.29)	<.0001
Histology type				
Lobular carcinoma vs. ductal	1.01 (0.76,1.34)	.94	1.03 (0.77,1.37)	.86
Other carcinoma vs. ductal	0.46 (0.22,0.97)	.041	0.72 (0.32,1.62)	.43
HER2				
Positive vs. negative	1.58 (1.31,1.91)	<.00001	1.34 (1.10,1.64)	.0043
TILs				
≥30% vs. <30%	0.88 (0.66,1.16)	.35	0.62 (0.46,0.83)	.0013
Overall mortality				
PMRT				
PMRT vs. no PMRT	0.79 (0.69,0.91)	.0013	0.78 (0.68,0.91)	<.001
Menopausal status/protocol[a]				
Post- vs. premenopausal	1.41 (1.23,1.63)	<.00001	1.43 (1.24,1.65)	<.00001
T-size				
21–50mm vs. ≤20mm	1.44 (1.23,1.69)	<.00001	1.25 (1.06,1.47)	.0074
≥51mm vs. ≤20mm	2.25 (1.81,2.79)	<.00001	1.75 (1.40,2.19)	<.00001
Positive nodes				
1–3 vs. none	1.11 (0.79,1.57)	.54	1.28 (0.90,1.82)	.18
≥4 vs. none	2.78 (1.98,3.91)	<.00001	2.85 (2.02,4.03)	<.00001
Malignancy grade[b]				
Grade 2 vs. grade 1	1.28 (1.07,1.54)	.0062	1.11 (0.93,1.34)	.25
Grade 3 vs. grade 1	1.67 (1.36,2.06)	<.00001	1.42 (1.12,1.80)	.0033
Histology type				
Lobular carcinoma vs. ductal	1.12 (0.89,1.42)	.34	1.11 (0.87,1.42)	.39
Other carcinoma vs. ductal	0.69 (0.41,1.17)	.17	0.86 (0.44,1.67)	.65
HER2				
Positive vs. negative	1.48 (1.25,1.75)	<.00001	1.31 (1.10,1.58)	.0032
TILs				
≥30% vs. <30%	0.84 (0.66,1.06)	.15	0.65 (0.50, 0.84)	<.001

*The multivariate analysis is stratified for estrogen receptor status (ER), and ER is therefore not listed among the variables in the table.
[a]According to study protocol, premenopausal women were treated with CMF and postmenopausal women with tamoxifen.
[b]Excluding 8 patients without tumor grade.

ER– tumors. The group of patients with ER–/high TILs BC showed an 11% improvement from PMRT (60% to 49%) as opposed to a 2% improvement (62% to 60%) for low TILs, but the interaction test was non-significant ($p = 0.71$) (Supplemental Figure 3(E–H)). The benefit of PMRT on the risk of LRR was not influenced by the level of TILs (Supplemental Figure 3(A–D)).

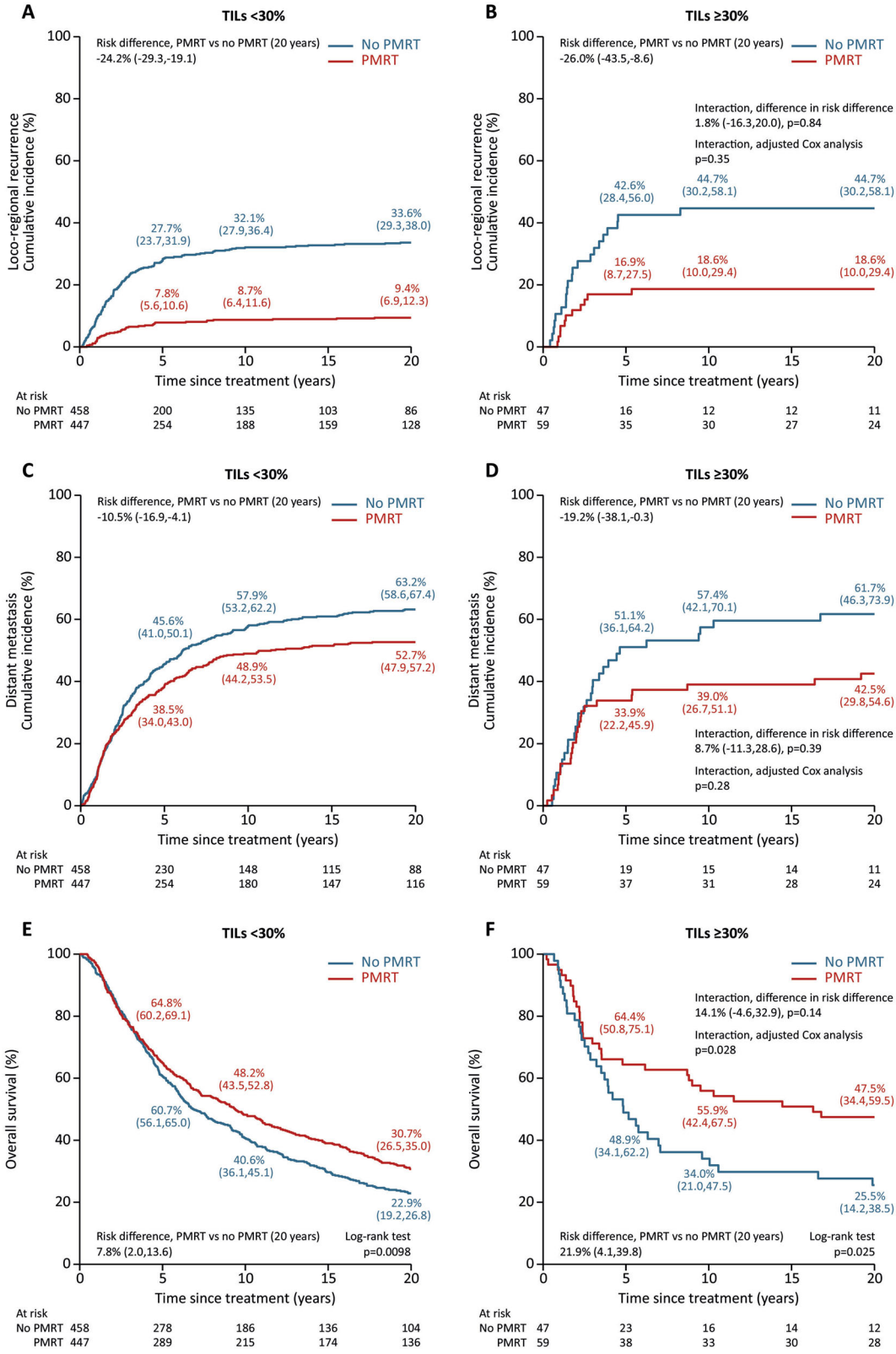

Figure 2. Cumulative incidence proportion plots for (A,B) risk of loco-regional recurrence (LRR) and (C,D) risk of distant metastasis (DM) and Kaplan–Meier plots for (E,F) overall survival (OS) for all 1,011 patients divided according to level of tumor-infiltrating lymphocytes (TILs) and stratified according to randomization to post-mastectomy radiotherapy (PMRT) or not (red – PMRT, blue – no PMRT). Actuarial risks at 5, 10 and 20 years and risk difference in actuarial risks at 20 years with 95% confidence intervals are provided, as well as *p*-values for Log Rank tests for Kaplan–Meier estimates. Interaction tests between TILs and PMRT are stated for both adjusted Cox analysis and risk differences.

When attempting to further divide the ER- patients into subtypes according to HER2 status, the positive association between high TILs and PMRT on OS was clearly seen in the ER–/HER2– group (Supplemental Figure 4(A–D)). The number of patients in each group was, nevertheless, very small.

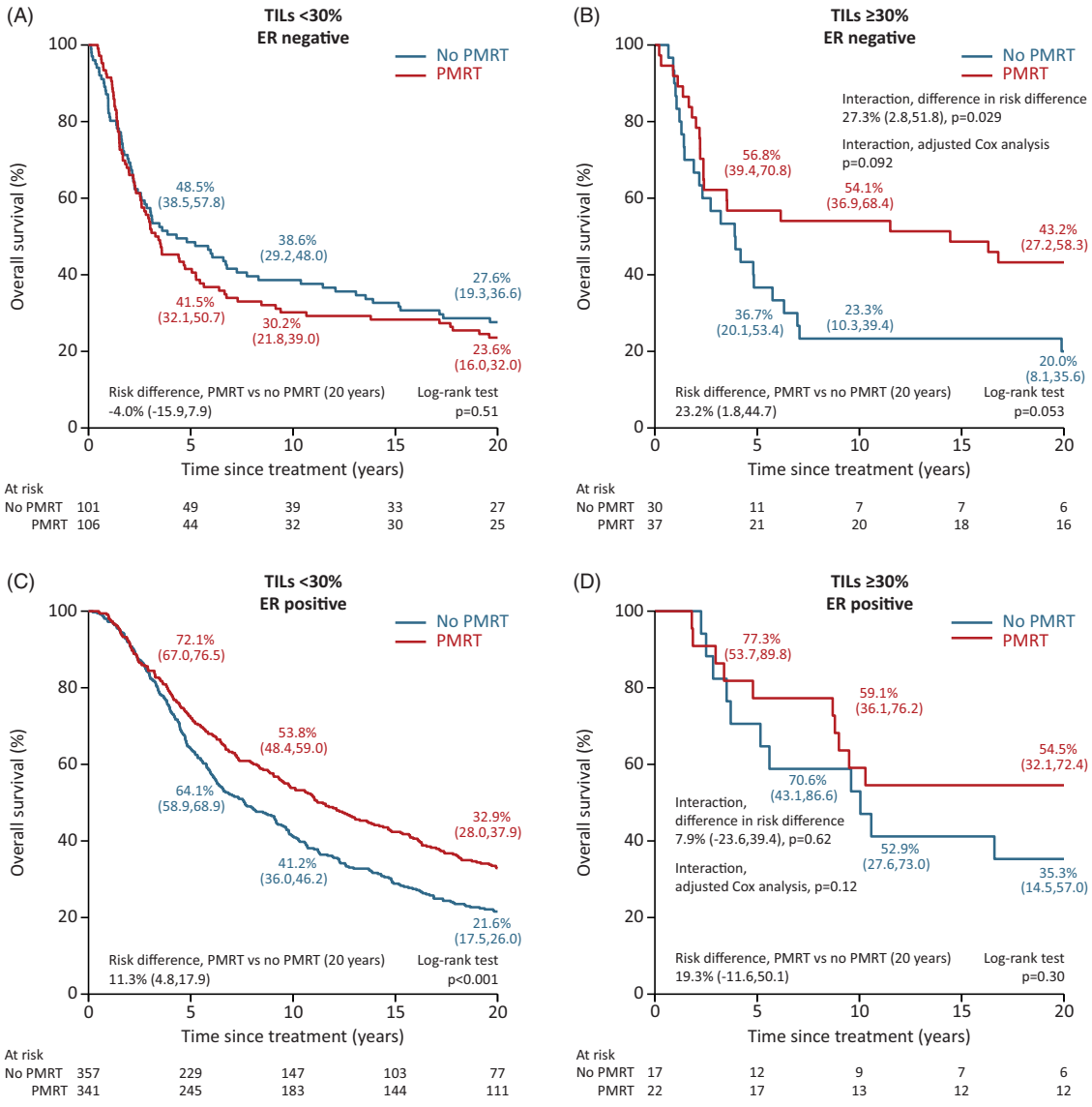

Figure 3. Kaplan–Meier plots for overall survival (OS) for (A,B) estrogen receptor (ER) negative and (C,D) ER-positive tumors divided according to level of tumor-infiltrating lymphocytes (TILs) and stratified according to randomization to post-mastectomy radiotherapy (PMRT) or not (red – PMRT, blue – no PMRT). Actuarial risks at 5, 10 and 20 years and risk difference in actuarial risks at 20 years with 95% confidence intervals are provided, as well as *p*-values for Log Rank tests and interaction tests between TILs and PMRT (for both adjusted Cox analysis and risk differences).

There was no significant interaction between ER status and menopausal status for any of the clinical endpoints, meaning that the postmenopausal, ER– patients (treated as part of the DBCG82bc trial with tamoxifen) did not have a significantly poorer outcome than the other patients, and adjusting for menopausal status (and hence systemic treatment) did not change the results.

Discussion

The present results showed that high TILs in pretreated BC tumor tissue were significantly associated with a lower risk of distant metastasis (DM) and improved OS in MVA. Furthermore, patients with high TILs in the pretreated tumor gained a significantly greater OS benefit from adjuvant PMRT than patients with low TILs; a trend was also seen for a reduction in risk of DM but not for the reduction of LRR. The association appeared especially pronounced among ER– tumors, primarily encompassing tumors of aggressive biology with a tendency for early DM spread.

The observed improved survival for high TILs patients cannot be explained by a better baseline survival, since a similar risk of overall death (23% *vs.* 26% for low and high TILs, respectively) and risk of DM (63% *vs.* 62% for low and high TILs, respectively) were observed among non-irradiated patients in the two TILs groups. This pattern was maintained in the group of ER– tumors, where non-irradiated patients also showed a similar prognosis in the two TILs groups. The association between TILs and PMRT may reflect an inherent difference in radio sensitivity related to the biology of the tumor, for which the presence of TILs could be merely a phenotypical feature of the specific biology and not directly related to the radio sensitivity. In this case, a similarly superior local control in high TILs as compared to low TILs patients would be expected after PMRT; but this was not the

case. Kovács *et al.* [9] saw a larger benefit from RT after BCS in terms of risk of IBTR in patients with low TILs in the SweBCT91RT trial, but a significant interaction between RT and TILs could not be proven. In our study, the LRR risk was not associated with TILs levels.

Accordingly, the observed survival gain from PMRT in the high TILs group cannot be explained as a consequence of better local control leading directly to a reduced risk of derived DM. The benefit of RT in reducing BC mortality without a cause-effect relationship between LRR and survival, but due to a systemic effect outside the treatment field ('abscopal' effect) has been discussed by Jatoi *et al.* [40] inspired by results from several clinical trials incl. the initial studies of the DBCG82bc cohort [29,30]. The underlying mechanism behind systemic effects of RT is suspected to involve activation of cytotoxic T-lymphocytes circulating through the body, reaching distant tumor deposits and leading to tumor regression [41]. Besides destroying tumor cells by a non-immunogenic cell death effectuated by direct damage to the DNA and indirect influence by free oxygen radicals, RT also induces immunogenic cell death. This is mediated through RT-induced release of 1) tumor-associated neo-antigens after local tumor destruction, and 2) damaged DNA gaining access to the cytosol through defect nucleic membranes in irradiated cells and thereby acting as powerful damage-associated molecular pattern (DAMP) activating the cGAS/STING pathway [42]. Both mechanisms lead to the priming of tumor-specific, cytotoxic T-lymphocytes in the draining lymph nodes [16,43] that may circulate and reach distant metastatic deposits.

The present results could reflect activation of RT-induced tumor-specific, immune response in tumors with high TILs leading to regression of distant micro-metastases outside the treatment field and subsequent improvement of OS. This effect may be accentuated in ER– tumors with a predilection for early DM. These findings are in line with previous findings in the DBCG82bc cohort [20] but adds valuable information by apparently identifying a group of ER–/high TILs tumors gaining survival benefit from PMRT. Results from further subdivision into ER– (non-luminal) subtypes were limited by the low number of patients in each group. Furthermore, the patients were treated before the introduction of anti-HER2 therapy, and the cohort is not optimal for drawing conclusions on a differential effect of TILs in HER2– vs. HER2+ patients.

The adverse prognostic effect of TILs according to ER status has previously been speculated to be related to resistance to endocrine therapy [5] and tamoxifen has been found to depress immunity by several mechanisms. Tamoxifen has for instance been described to induce a shift from Th1 toTh2 polarization of CD4+ cells impairing the antitumor response [44]; a mechanism described to be independent of the estrogen receptor. Furthermore, tamoxifen may impair the differentiation and maturation of dendritic cells interfering with T-cell proliferation [45]. In this study, tamoxifen was, according to the study protocol, given to all postmenopausal women regardless of ER status. This limits the possibility of evaluating a possible influence of tamoxifen's immunomodulating effect on the associations between ER status, PMRT and TILs.

From the present evaluation of TILs, it cannot be deducted, if the observed differences in clinical outcome are related to the presence of specific immune cells (e.g., cytotoxic T-cells) or variabilities in cGAS/STING expression. Neither can it be determined, if results are influenced by variation in the composition of immune cells between ER– and ER + tumors. Ongoing studies on the DBCG82bc cohort address these questions.

Several large clinical studies have shown a disease-free survival benefit in patients with extended nodal irradiation [46,47], and an OS benefit with irradiation of the internal mammary nodes (IMN) has been proven in the DBCG-IMN1 cohort [25]. The PMRT in our study was delivered to the chest wall and lymph nodes after removal of the whole mammary gland with residues of tumor tissue likely to be minute. The observed association between TILs and RT may, therefore, be mediated through irradiation of the regional lymph nodes instead of the chest wall. An examination of TILs in the IMN cohort has recently been initiated to further explore this association.

The present results beg the intriguing question of whether 'cold tumors' with few TILs can become 'hot tumors' and gain the same survival benefits from PMRT. Checkpoint inhibitor antagonists may assist in pushing the balance toward immune-competence, and the possible synergistic effect between RT and checkpoint inhibitors is being extensively studied (reviewed in [48,49]). Immunotherapy reactivates, however, the adaptive immune response and is most effective in patients with an already preexisting immune response including the presence of TILs. 'Kickstarting' the immune-response and turning 'cold tumors' into 'hot tumors' is currently being examined, and e.g., delivery of a low dose RT (8 Gy/3 fractions) in combination with immunotherapy has proven successful in preclinical studies [50].

The cut off of 30% to distinguish 'cold tumors' with low TILs vs 'hot tumors' with high TILs was chosen because it represented the cut off with the best interobserver agreement, but it was also found to deliver the most optimal associations with clinical endpoints and significant interactions between PMRT and TILs as compared to a 20% or 40% cut off. A similar cut-off for TILs has previously been used by Loi *et al.* [2].

A limitation to our study is that patients were treated with surgical techniques and systemic treatment not comparable to contemporary treatment, which may compromise the external validity of the size of the effect on BC patients today. Though the absolute survival benefits are likely smaller in currently treated patients due to more aggressive systemic treatment, the biological association between TILs, ER status and PMRT is likely unaltered.

Another limitation is that even though more than 1000 patients were included in the study, only 106 patients harbored a high level of TILs, which led to restrictions in the statistical power in some subgroups due to few patients in each group.

The strengths of the study are the randomized design of the DBCG82bc trial, the long clinical follow-up and a large number of events. This renders the cohort optimal for hypothesis-generating studies as similar recurrence rates are not observed in present days. The TILs evaluation on routinely processed, whole slide HE-sections is further robust and easily applicable in a daily clinical setting. The evaluation is also not affected by age of the blocks as may be the case with gene expression profiling and IHC staining.

In conclusion, BC patients with a high level of TILs in pretreatment tumor tissue gained a significantly larger OS benefit from adjuvant PMRT; a trend was also seen for high TILs and a reduced risk of DM after PMRT. The association was especially strong among ER– tumors. These findings may indicate that PMRT triggers a local immune response inducing a systemic effect outside the treatment field, e.g., by eliminating distant metastatic deposits. Further studies are needed to understand the mechanism behind the association and to explore if patients with 'cold tumors' (low TILs) can be turned into 'hot tumors' (high TILs) to obtain the same superior survival effect by PMRT.

Disclosure statement

TT, JA, JO are listed as co-inventors on an application for an international patent number WO2013132354A3, titled 'Gene signatures associated with the efficacy of post-mastectomy radiotherapy in breast cancer.'

Funding

The study is fully supported by grants from the Danish Cancer Society (R146-A-9519 and R148-A10067-16-S46).

ORCID

Trine Tramm http://orcid.org/0000-0003-3894-4552
Jan Alsner http://orcid.org/0000-0002-5395-3193
Jens Overgaard http://orcid.org/0000-0002-0814-8179

References

[1] Stanton SE, Adams S, Disis ML. Variation in the incidence and magnitude of tumor-infiltrating lymphocytes in breast cancer subtypes: a systematic review. JAMA Oncol. 2016;2(10):1354–1360.
[2] Loi S, Drubay D, Adams S, et al. Tumor-Infiltrating lymphocytes and prognosis: a pooled individual patient analysis of early-stage triple-negative breast cancers. J Clin Oncol. 2019;37(7):559–569.
[3] Ibrahim EM, Al-Foheidi ME, Al-Mansour MM, et al. The prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancer: a meta-analysis. Breast Cancer Res Treat. 2014;148(3):467–476.
[4] Salgado R, Denkert C, Campbell C, et al. Tumor-infiltrating lymphocytes and associations with pathological complete response and event-free survival in HER2-positive early-stage breast cancer treated with lapatinib and trastuzumab: a secondary analysis of the neoALTTO trial. JAMA Oncol. 2015;1(4):448–454.
[5] Denkert C, von Minckwitz G, Darb-Esfahani S, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol. 2018;19(1):40–50.
[6] Gonzalez-Ericsson PI, Stovgaard ES, Sua LF, et al. The path to a better biomarker: application of a risk management framework for the implementation of PD-L1 and TILs as immuno-oncology biomarkers in breast cancer clinical trials and daily practice. J Pathol. 2020;250(5):667–684.
[7] Goto T. Radiation as an in situ auto-vaccination: current perspectives and challenges. Vaccines. 2019;7:100.
[8] Sato H, Okonogi N, Nakano T. Rationale of combination of anti-PD-1/PD-L1 antibody therapy and radiotherapy for cancer treatment. Int J Clin Oncol. 2020;25(5):801–809.
[9] Kovács A, Tullberg AS, Rönnerman EW, et al. Effect of radiotherapy after breast-conserving surgery depending on the presence of tumor-infiltrating lymphocytes: a long-term follow-up of the SweBCG91RT randomized trial. J Clin Oncol. 2019;37(14):1179–1187.
[10] Stenmark Tullberg A, Puttonen HAJ, Sjöström M, et al. Immune infiltrate in the primary tumor predicts effect of adjuvant radiotherapy in breast cancer; results from the randomized SweBCG91RT trial. Clin Cancer Res. 2021;27(3):749–758.
[11] Ho AY, Wright JL, Blitzblau RC, et al. Optimizing radiation therapy to boost systemic immune responses in breast cancer: a critical review for breast radiation oncologists. Int J Radiat Oncol Biol Phys. 2020;108(1):227–241.
[12] Ye JC, Formenti SC. Integration of radiation and immunotherapy in breast cancer – treatment implications. Breast. 2018;38:66–74.
[13] Colton M, Cheadle EJ, Honeychurch J, et al. Reprogramming the tumour microenvironment by radiotherapy: implications for radiotherapy and immunotherapy combinations. Radiat Oncol. 2020;15(1):254.
[14] Van den Ende T, van den Boorn HG, Hoonhout NM, et al. Priming the tumor immune microenvironment with chemo(radio)therapy: a systematic review across tumor types. Biochim Biophys Acta Rev Cancer. 2020;1874(1):188386.
[15] Rudqvist N-P, Pilones KA, Lhuillier C, et al. Radiotherapy and CTLA-4 blockade shape the TCR repertoire of tumor-infiltrating T cells. Cancer Immunol Res. 2018;6(2):139–150.
[16] Rodríguez-Ruiz ME, Vanpouille-Box C, Melero I, et al. Immunological mechanisms responsible for radiation-induced abscopal effect. Trends Immunol. 2018;39(8):644–655.
[17] Darby SC, Ewertz M, McGale P, et al. Risk of ischemic heart disease in women after radiotherapy for breast cancer. N Engl J Med. 2013;368(11):987–998.
[18] Milo MLH, Thorsen LBJ, Johnsen SP, et al. Risk of coronary artery disease after adjuvant radiotherapy in 29,662 early breast cancer patients: a population-based Danish breast cancer group study. Radiother Oncol. 2021;157:106–113.
[19] Grantzau T, Mellemkjaer L, Overgaard J. Second primary cancers after adjuvant radiotherapy in early breast cancer patients: a national population based study under the Danish breast cancer cooperative group (DBCG). Radiother Oncol. 2013;106(1):42–49.
[20] Kyndi M, Sørensen FB, Knudsen H, et al. Estrogen receptor, progesterone receptor, HER-2, and response to postmastectomy radiotherapy in high-risk breast cancer: the Danish breast cancer cooperative group. J Clin Oncol. 2008;26(9):1419–1426.
[21] Braunstein LZ, Taghian AG, Niemierko A, et al. Breast-cancer subtype, age, and lymph node status as predictors of local recurrence following breast-conserving therapy. Breast Cancer Res Treat. 2017;161(1):173–179.
[22] Tseng YD, Uno H, Hughes ME, et al. Biological subtype predicts risk of locoregional recurrence after mastectomy and impact of postmastectomy radiation in a large national database. Int J Radiat Oncol Biol Phys. 2015;93(3):622–630.
[23] Fitzal F, Filipits M, Fesl C, et al. PAM-50 predicts local recurrence after breast cancer surgery in postmenopausal patients with ER+/HER2–disease: results from 1204 patients in the randomized ABCSG-8 trial. Br J Surg. 2021;108(3):308–314.
[24] Poortmans PMP, Arenas M, Livi L. Over-irradiation. Breast. 2017;31:295–302.

[25] Thorsen LBJ, Offersen BV, Danø H, et al. DBCG-IMN: a Population-Based cohort study on the effect of internal mammary node irradiation in early Node-Positive breast cancer. J Clin Oncol. 2016;34(4):314–320.
[26] Offersen BV, Alsner J, Nielsen HM, et al. Hypofractionated versus standard fractionated radiotherapy in patients with early breast cancer or ductal carcinoma in situ in a randomized phase III trial: the DBCG HYPO trial. J Clin Oncol. 2020;38(31):3615–3625.
[27] Haviland JS, Owen JR, Dewar JA, et al. The UK standardisation of breast radiotherapy (START) trials of radiotherapy hypofractionation for treatment of early breast cancer: 10-year follow-up results of two randomised controlled trials. Lancet Oncol. 2013; 14(11):1086–1094.
[28] Tramm T, Mohammed H, Myhre S, et al. Development and validation of a gene profile predicting benefit of postmastectomy radiotherapy in patients with high-risk breast cancer: a study of gene expression in the DBCG82bc cohort. Clin Cancer Res. 2014; 20(20):5272–5280.
[29] Overgaard M, Jensen M-B, Overgaard J, et al. Postoperative radiotherapy in high-risk postmenopausal breast-cancer patients given adjuvant tamoxifen: Danish breast cancer cooperative group DBCG 82c randomised trial. Lancet. 1999;353(9165):1641–1648.
[30] Overgaard M, Hansen PS, Overgaard J, et al. Postoperative radiotherapy in high-risk premenopausal women with breast cancer who receive adjuvant chemotherapy. Danish breast cancer cooperative group 82b trial. N Engl J Med. 1997;337(14):949–955.
[31] Overgaard M, Nielsen HM, Overgaard J. Is the benefit of postmastectomy irradiation limited to patients with four or more positive nodes, as recommended in international consensus reports? A subgroup analysis of the DBCG 82 b&c randomized trials. Radiother Oncol. 2007;82(3):247–253.
[32] Nielsen HM, Overgaard M, Grau C, et al. Study of failure pattern among high-risk breast cancer patients with or without postmastectomy radiotherapy in addition to adjuvant systemic therapy: long-term results from the Danish breast cancer cooperative group DBCG 82 b and c randomized studies. J Clin Oncol. 2006; 24(15):2268–2275.
[33] Wolff AC, Hammond MEH, Schwartz JN, et al. American society of clinical oncology/college of American pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Arch Pathol Lab Med. 2007;131(1):18–43.
[34] Salgado R, Denkert C, Demaria S, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an international TILs working group 2014. Ann Oncol. 2015; 26(2):259–271.
[35] McShane LM, Altman DG, Sauerbrei W, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst. 2005;97(16):1180–1184.
[36] Denkert C, Wienert S, Poterie A, et al. Standardized evaluation of tumor-infiltrating lymphocytes in breast cancer: results of the ring studies of the international immuno-oncology biomarker working group. Mod Pathol. 2016;29(10):1155–1164.
[37] Denkert C, von Minckwitz G, Brase JC, et al. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. J Clin Oncol. 2015;33(9):983–991.
[38] Gisev N, Bell JS, Chen TF. Interrater agreement and interrater reliability: key concepts, approaches, and applications. Res Social Adm Pharm. 2013;9(3):330–338.
[39] Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977;33(1):159–174.
[40] Jatoi I, Benson JR, Kunkler I. Hypothesis: can the abscopal effect explain the impact of adjuvant radiotherapy on breast cancer mortality? NPJ Breast Cancer. 2018;4:8.
[41] Demaria S, Ng B, Devitt ML, et al. Ionizing radiation inhibition of distant untreated tumors (abscopal effect) is immune mediated. Int J Radiat Oncol Biol Phys. 2004;58(3):862–870.
[42] Mackenzie KJ, Carroll P, Martin C-A, et al. cGAS surveillance of micronuclei links genome instability to innate immunity. Nature. 2017;548(7668):461–465.
[43] Galluzzi L, Buqué A, Kepp O, et al. Immunogenic cell death in cancer and infectious disease. Nat Rev Immunol. 2017;17(2): 97–111.
[44] Li B, Li Y, Wang X-Y, et al. Profile of differentially expressed intratumoral cytokines to predict the immune-polarizing side effects of tamoxifen in breast cancer treatment. Am J Cancer Res. 2015; 5(2):726–737.
[45] Nalbandian G, Paharkova-Vatchkova V, Mao A, et al. The selective estrogen receptor modulators, tamoxifen and raloxifene, impair dendritic cell differentiation and activation. J Immunol. 2005; 175(4):2666–2675.
[46] Whelan TJ, Olivotto IA, Parulekar WR, et al. Regional nodal irradiation in early-stage breast cancer. N Engl J Med. 2015;373(4): 307–316.
[47] Poortmans PM, Collette S, Kirkove C, et al. Internal mammary and medial supraclavicular irradiation in breast cancer N. N Engl J Med. 2015;373(4):317–327.
[48] Levy A, Chargari C, Marabelle A, et al. Can immunostimulatory agents enhance the abscopal effect of radiotherapy? Can immunostimulatory agents enhance the abscopal effect of radiotherapy? Eur J Cancer. 2016;62:36–45.
[49] Naghavi AO, Johnstone PAS, Kim S. Clinical trials exploring the benefit of immunotherapy and radiation in cancer treatment: a review of the past and a look into the future. Curr Probl Cancer. 2016;40(1):38–67.
[50] Dewan MZ, Galloway AE, Kawashima N, et al. Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. Clin Cancer Res. 2009;15(17):5379–5388.