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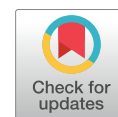
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Clinical Investigation

# Tumor-Infiltrating Lymphocytes in Low-Risk Patients With Breast Cancer Treated With Single-Dose Preoperative Partial Breast Irradiation



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Received Jun 2, 2020. Accepted for publication Dec 7, 2020.

**Purpose:** Preoperative partial breast irradiation (PBI) has the potential to induce tumor regression. We evaluated the differences in the numbers of preirradiation tumor infiltrating lymphocytes (TILs) between responders and nonresponders after preoperative PBI in low-risk patients with breast cancer. Furthermore, we evaluated the change in number of TILs before and after irradiation.

**Methods and Materials:** In the prospective ABLATIVE study, low-risk patients with breast cancer underwent treatment with single-dose preoperative PBI (20 Gy) to the tumor and breast-conserving surgery after 6 or 8 months. In the preirradiation diagnostic biopsy and postirradiation resection specimen, numbers of TILs in 3 square regions of 450 × 450 μm were counted manually. TILs were visualized with CD3, CD4, and CD8 immunohistochemistry. Differences in numbers of preirradiation TILs between responders and nonresponders were tested using Mann-Whitney *U* test. Responders were defined as pathologic complete or near-complete response, and nonresponders were defined “as all other response.” Changes in numbers of TILs after preoperative PBI was evaluated with the Wilcoxon signed rank test.

**Results:** Preirradiation tissue was available from 28 patients, postirradiation tissue from 29 patients, resulting in 22 pairs of preirradiation and postirradiation tissue. In these 35 patients, 15 had pathologic complete response (43%), 11 had a near-complete response (31%), 7 had a partial response (20%), and 2 had stable disease (6%). The median numbers of CD3<sup>+</sup> TILs, CD4<sup>+</sup> TILs, and CD8<sup>+</sup> TILs in the preirradiation tumor tissue were 49 (interquartile range [IQR], 36-80), 45 (IQR, 28-57), and 19 (IQR, 8-35), respectively. The number of preirradiation TILs did not differ significantly between responders and

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This study received funding from Pink Ribbon (2012.WO22.C154). The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and the decision to submit the manuscript for publication.

Disclosures: none.

Data sharing statement: Research data are not available at this time.

**Acknowledgments**—Ron Koelemij, Annemiek Doeksen, Maartje F Sier, Arjen J Witkamp, Thijs van Dalen, Elsken van der Wall, and Iris E van Dam.

nonresponders. The median numbers of CD3<sup>+</sup> TILs, CD4<sup>+</sup> TILs, and CD8<sup>+</sup> TILs in postirradiation tumor tissue were 17 (IQR, 13-31), 26 (IQR, 16-35), and 7 (IQR, 5-11), respectively.

**Conclusions:** After preoperative PBI in this limited cohort, the number of TILs in tumor tissue decreased. No differences in numbers of preirradiation TILs between responders and nonresponders were observed. © 2020 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

## Introduction

Preoperative partial breast irradiation (PBI) has the potential to induce tumor regression in patients with breast cancer.<sup>1</sup> In our previous study (ABLATIVE, ClinicalTrials.gov identifier: NCT06863301) of a single dose (20 Gy) of preoperative PBI, 15 of 36 low-risk patients with breast cancer resulted in a pathologic complete response after an interval of 6 to 8 months between irradiation and breast-conserving surgery.<sup>2</sup> Complete tumor regression after preoperative PBI could allow omission of breast surgery in future patients with no clinical evidence of residual disease.<sup>3,4</sup> To assess which patients will achieve or have achieved pathologic complete response (pCR), adequate response assessment is eminent.

Response assessment during standard preoperative systemic treatment currently consists of magnetic resonance imaging or <sup>18</sup>F-fluorodeoxyglucose positron emission tomography–computed tomography (PET/CT), or both. Several studies have shown that the predictive value of both magnetic resonance imaging and PET/CT for pathologic response is insufficient to identify patients in whom surgery after preoperative systemic treatment (PST) can be omitted.<sup>5-7</sup>

To increase the predictive value of response assessment after preoperative systemic treatment or radiation therapy, in the assessment of immune infiltrates, so-called tumor infiltrating lymphocytes (TILs) have been proposed as a biomarker.<sup>8-11</sup> Increased numbers of TILs in resection specimens of patients with triple-negative breast cancer treated with PST have been associated with improved outcome, such as disease-free survival and overall survival.<sup>8</sup> The explanation for the association between the number of TILs and improved clinical outcome lies within the activation of the immune system after PST, which can result in the inhibition of tumor growth and induction of immunogenic cell death.<sup>12-14</sup> The number of TILs can be evaluated as a representation of the activation of the immune system. However, TILs cannot be identified in all patients, thus complicating the investigation of TILs as a biomarker.<sup>15,16</sup> The important types of TILs in patients with breast cancer whom we studied are CD3<sup>+</sup> TILs, CD4<sup>+</sup> TILs, and CD8<sup>+</sup> TILs. The expression of CD3 is crucial for the activation of T cells in an antitumor response, activation of CD4 directly activates CD8<sup>+</sup> T cells and leads to the production of tumor necrosis factor- $\alpha$ .<sup>17-19</sup> CD8 is expressed on cytotoxic T cells, and it increases sensitivity of the T cell to the presented antigen.<sup>17,20</sup>

In this study, we assessed TILs before and after preoperative PBI in low-risk patients, and we evaluated the differences in numbers of preoperative TILs between responders and nonresponders as a possible biomarker for future response monitoring.

## Methods and Materials

### Patient selection

The preirradiation diagnostic core needle biopsies and postirradiation resection specimens of low-risk patients with luminal breast cancer included in the single arm ABLATIVE trial of single-dose preoperative PBI were evaluated.<sup>2</sup> The ABLATIVE trial was approved by the institutional review board of the UMC Utrecht, the Netherlands, and registered at [ClinicalTrials.gov](http://ClinicalTrials.gov) (NCT06863301). After providing informed consent, patients with unifocal ductal or mucinous invasive breast cancer with a maximum diameter of 3 cm, ER-positive and HER2-negative tumor, a negative sentinel lymph node biopsy, and no indication for preoperative chemotherapy or immunotherapy were enrolled. Participating patients received a single ablative radiation therapy dose of 20 Gy to the tumor and 15 Gy to the breast tissue within 2 cm of the tumor. Patients underwent breast-conserving surgery after an interval of 6 or 8 months after preoperative PBI. The pathologic response after preoperative PBI was assessed using the European Society of Breast Cancer Specialists criteria according to the national guidelines.<sup>21,22</sup> The possible responses were (1) pCR, (2) near pCR (<10% residual disease), (3) partial response (10%-50% residual disease), (4) stable disease (>50% residual disease), or (5) no evidence of response. Thirty-six women were included in the clinical trial, but for 1 patient no additional preirradiation and postirradiation tumor tissue could be retrieved. For 22 patients, both the diagnostic biopsy and the resection specimen were available; for 6 patients, only the preirradiation diagnostic biopsy was available; and for 7 patients, only the postirradiation resection specimen was available.

### Assessment of tumor infiltrating lymphocytes

Consecutive slides of 4  $\mu$ m were obtained from formalin-fixed paraffin-embedded tissue blocks of the diagnostic tumor biopsy and resection specimens. These slides were

prepared for immunohistochemical (IHC) staining for CD3, CD4, and CD8, using rabbit anti-CD3 polyclonal antibody (DAKO, A0452, dilution 1:100; Glostrup, Denmark), rabbit anti-CD4 monoclonal antibody (Cellmarque, 104R-16, dilution 1:20; Rocklin, CA), and mouse anti-CD8 monoclonal antibody (DAKO, M7103, dilution 1:100), respectively. If available, an additional slide was stained with hematoxylin and eosin (HE). All slides were digitalized with a NanoZoomer-XR digital slide scanner (Hamamatsu, Hamamatsu City, Japan) at original magnification  $\times 40$ .

The preirradiation and postirradiation slides were assessed separately. The clinical information and histopathologic reports were available during annotation of representative tumor areas. This annotation was performed by a researcher (M.D.) and an experienced breast pathologist on HE-stained slides and then copied to the IHC-stained slides. When no HE-stained slide was available, the first annotation was performed on CD3-stained slides. The tumor was annotated on the preirradiation slides, and the irradiated tumor and area of tumor regression were annotated on the postirradiation slides. The irradiated tumor and area of regression were identified by reactive changes, such as scarlike fibrosis and iron-loaded macrophages. If distinction between the area of regression and surrounding stroma was not clear, stroma with the same density as the definitive area of regression was included in the annotation. After tumor or area of regression annotation, 3 square fields of  $450 \times 450 \mu\text{m}$  were selected randomly in all slides, and the number of TILs was quantified manually by dotting each lymphocyte in these fields. TIL assessment was performed blinded to clinical information and histopathologic reports.

### Statistical analysis

Pathologic response was grouped to responders (defined as pCR and near-pCR) and nonresponders (defined as partial response, stable disease, and no evidence of response). The number of TILs was expressed as the mean of the 3 selected squares and calculated for both the diagnostic biopsy and resection specimen. The number of preirradiation and postirradiation TILs and change in number of TILs were presented using median and interquartile range (IQR). Differences in numbers of preirradiation TILs between responders and nonresponders were tested using Mann-Whitney *U* test. Differences in numbers of TILs between preirradiation and postirradiation were tested using Wilcoxon signed rank test. Statistical tests were 2-sided and performed at a significance level of 0.05. Statistical analysis was performed using IBM SPSS Statistics for Windows, version 25 (IBM, Armonk, NY).

### Results

Of the 35 analyzed low-risk patients with breast cancer, the median age was 64 years (range, 51-78 years) and the

median tumor size was 13 mm (range, 5-20 mm; Table 1). Of the total of 35 patients, 15 patients achieved pCR (43%), 11 patients achieved near pCR (31%), 7 patients achieved partial response (20%), and 2 patients had stable disease (6%). Of the 28 preirradiation samples, 13 patients achieved pCR (46%), 9 patients achieved near pCR (32%), 5 patients achieved partial response (18%), and 1 patient had stable disease (4%). Of the 29 postirradiation samples, 12 patients achieved pCR (41%), 10 patients achieved near pCR (35%), 6 patients achieved partial response (21%), and 1 patient had stable disease (3%). In some cases, only the preirradiation (6 cases) or postirradiation (7 cases) slides were available, because no tumor material was left for IHC staining after the necessary clinical pathology assessment. In 5 cases the preirradiation slide did not contain enough tumor material to select 3 square fields of  $450 \times 450 \mu\text{m}$ ; therefore, fewer fields were selected. Because of technically inadequate staining, not all IHC staining could be assessed for every case.

In the preirradiation slides the median number of CD3<sup>+</sup> TILs was 49 (IQR, 36-80), of CD4<sup>+</sup> TILs 45 (IQR, 28-57), and of CD8<sup>+</sup> TILs 19 (IQR, 8-35; Fig. 1). For responders and nonresponders, the median number of CD3<sup>+</sup> was 57 and 45 (*P* = .74), the median number of CD4<sup>+</sup> 42 and 50 (*P* = .98), and the median number of CD8<sup>+</sup> was 19 and 16 (*P* = .48), respectively (Fig. 2). In the postirradiation

**Table 1** Patient and tumor characteristics of 35 low-risk patients with breast cancer studied for the association between tumor infiltrating lymphocytes and pathologic response after preoperative partial breast irradiation

Characteristics	Data
Median tumor diameter, mm (range)*	13 (5-20)
Median age, y (range)	64 (51-78)
Bloom-Richardson grade, n (%)	
1	24 (69)
2	9 (26)
3	1 (3)
Not assessable†	1 (3)
Histology type, n (%)	
Ductal	34 (97)
Mucinous	1 (3)
Pathologic response, n (%)	
Pathologic complete response	15 (43)
Near complete response (<10% residual tumor cells)	11 (31)
Partial response (10%-50% residual tumor cells)	7 (20)
Stable disease (>50% residual tumor cells)	2 (6)
No response	0 (0)

\* Tumor diameter as assessed on magnetic resonance imaging.

† Not assessable because of small tumor biopsy.

slides, the median number of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> TILs was 17 (IQR, 13-31), 26 (IQR, 16-35), and 7 (IQR, 5-11), respectively (Fig. 1).

In the 22 patients in whom both the preirradiation and postirradiation number of TILs could be assessed, a statistically significant decrease in TILs was observed. In these 22 patients, the median preirradiation number of CD3<sup>+</sup> TILs was 45 (IQR, 33-79), and median postirradiation number of TILs 16 (IQR, 12-22), for CD4<sup>+</sup> TILs the numbers were 44 (IQR, 30-55) and 25 (IQR, 13-35), respectively, and for CD8<sup>+</sup> TILs the numbers were 17 (IQR, 7-37) and 6 (IQR, 5-9), respectively. A median decrease of 69% ( $P = .002$ ) was observed for CD3<sup>+</sup>, a median decrease of 27% ( $P = .003$ ) was observed for CD4<sup>+</sup>, and a median decrease of 74% ( $P = .004$ ) was observed for CD8<sup>+</sup>.

## Discussion

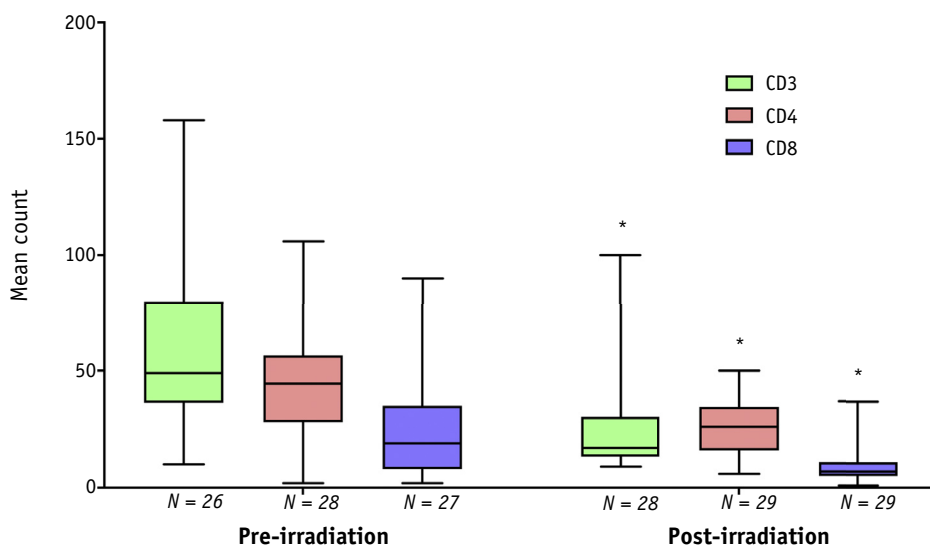
TILs could be clearly identified in all low-risk patients with breast cancer, both before and after irradiation (Fig. 3). We observed no differences in the number of preirradiation TILs between responders and nonresponders.

We observed a large range in the number of preirradiation and postirradiation TILs, with the largest range in the number of CD3<sup>+</sup> lymphocytes. Similarly, a large range in pretreatment TILs in patients with breast cancer treated with PST has been reported. Denkert et al<sup>23</sup> evaluated the percentage of intratumoral and stromal TILs before PST of cT1-3N0-2M0 breast cancer.<sup>23</sup> In core biopsy specimens of 1058 patients, they observed tumors without any TILs and tumors with >50% TILs. In a study that evaluated CD8<sup>+</sup> TILs in the resection specimen of 1334 patients with breast cancer (pT1-

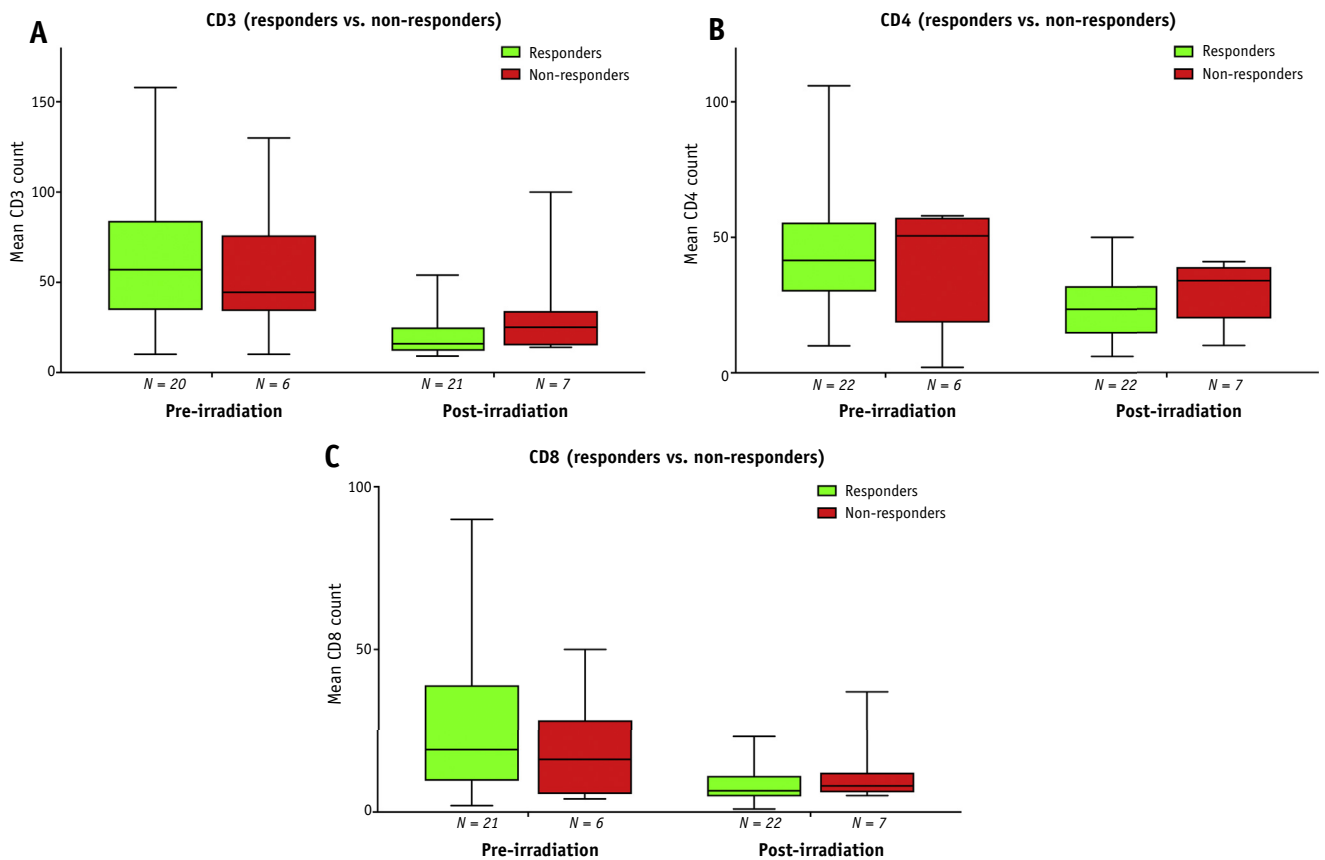
2N0-2M0) after primary surgery within the Nottingham Tenovus Primary Breast Carcinoma series, a median of 11 TILs was observed in a field of 0.28 mm<sup>2</sup> (IQR, 2-34).<sup>20</sup> This number of TILs is comparable to the median of 19 CD8<sup>+</sup> TILs (IQR, 8-35) in the preirradiation tumor tissue within our study, despite the low-risk patients in our study and in contrast to the more advanced disease in the Nottingham series. Kovács et al<sup>11</sup> evaluated a more comparable group of patients with early-stage breast cancer; they found that patients with luminal A and B type tumors had the lowest percentage of TILs.

The observed decrease in number of TILs after preoperative irradiation was higher than in previous studies of preoperative systemic treatment of locally advanced and inflammatory breast cancer.<sup>15,24</sup> In addition to the different treatment approach, this could be attributed to the lower number of preirradiation TILs and longer interval of up to 8 months between irradiation and postirradiation assessment of TILs in our study. This approach resulted in tumor regression after an ablative dose of radiation therapy, including fewer vital tumor cells for immune cells to respond to.

Pretreatment TILs have been shown to be a significant predictor of pathologic complete response and prolonged survival after PST in breast cancer.<sup>10,20,23</sup> In our small series, we did not observe an association between pretreatment TILs and pathologic response. Most of the affirmative studies on TILs during PST included patients with a more advanced disease stage than in the present study. In addition, many studies reported the predictive value of pretreatment TILs for response to PST in triple-negative breast cancer, and not in ER<sup>+</sup> breast cancers.<sup>25-27</sup> Moreover, all cases in our study, except for one, had a Bloom-Richardson grade 1 or 2 tumor. Low-grade tumors have been reported to have lower numbers of TILs than high-grade tumors



**Fig. 1.** Numbers of preirradiation and postirradiation (6 to 8 months after irradiation) tumor-infiltrating lymphocytes of patients with breast cancer treated with preoperative partial breast irradiation. \* $P < .05$  for difference with preirradiation number of tumor-infiltrating lymphocytes.



**Fig. 2.** Numbers of preirradiation and postirradiation (6 to 8 months after irradiation) tumor-infiltrating lymphocytes in low-risk patients with breast cancer treated with preoperative partial breast irradiation according to pathologic response. No significant differences in numbers of preirradiation tumor-infiltrating lymphocytes between responders and nonresponders were found.

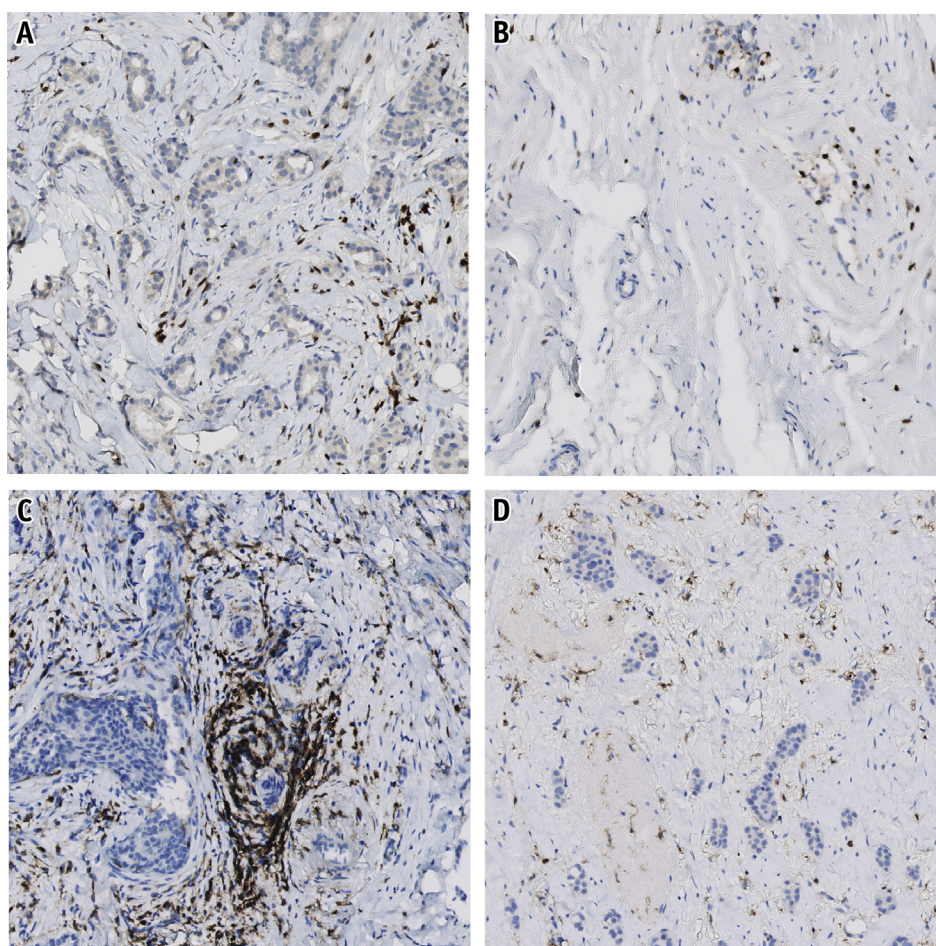
have,<sup>11,28</sup> which could also explain why we were not able to demonstrate an association between pretreatment TILs and pathologic response.

A strength of the current study is the availability of both preirradiation and postirradiation slides for the same patients, enabling us to assess the effect of irradiation on the number of TILs. Second, the number of TILs was assessed through several different IHC stainings that highlight TIL subtypes and that can help us in understanding the contribution of the different types of immune cells to respond to preoperative PBI. Although a significant decrease in TILs after preoperative PBI was observed, we could not differentiate between responders and nonresponders using the number of preoperative TILs, an important step in the ultimate treatment de-escalation (ie, omission of surgery). Nevertheless, it is remarkable that even after the long interval of 6 to 8 months after preoperative PBI, TILs were still observed in all cases. In several publications, TILs were not observed in cases achieving pCR after PST.<sup>15,16</sup> The TILs international working group has recently encouraged the evaluation of posttreatment TILs in a research setting, especially in the case of pCR.<sup>13</sup>

A limitation of the present study is that it was designed as a feasibility study for the novel treatment option of

single-dose preoperative PBI; therefore, it was not powered on finding predictors for treatment response. Furthermore, not all biopsy and resection specimens could be retrieved and evaluated, which decreased the already limited sample size. However, we assume that the missing samples were not associated with the pathologic response, because the percentage of responders and nonresponders was not different between the entire group of patients, the group of patients with a preirradiation sample available, and the group of patients with a postirradiation sample available. Therefore, the missing samples are presumably at random, and they will not affect the interpretation of our results. Nonetheless, a larger number of available slides could have improved the differentiation between responders and nonresponders, and we recommend further evaluation of TILs as a biomarker in future larger cohorts. Furthermore, as the preirradiation biopsy was performed for diagnostic purposes, only a single biopsy specimen was taken. Only the single biopsy specimen could be assessed, which could have led to overestimation or underestimation of the number of TILs, because no purposeful sampling has been performed.<sup>29</sup> The present study could also have benefitted from additional biopsies of the irradiated tumor a few weeks after irradiation, to better assess the acute immune





**Fig. 3.** Immunohistochemical positive staining of lymphocytes in low-risk patients with breast cancer. (A) Preirradiation CD3 staining. (B) Postirradiation CD3 staining in a patient with pathologic complete response (8 months after irradiation). (C) Preirradiation CD4 staining. (D) Postirradiation CD4 staining in a patient with partial response (6 months after irradiation).

response because the acute response has faded at 6 to 8 months after preoperative PBI. It could be hypothesized that tumors with a more extensive acute cellular immune response have a higher chance of achieving pCR, which might be better assessable 6 to 8 weeks after irradiation than the currently used 6 to 8 months. These additional biopsies were not performed to avoid imposing this additional burden on participating patients. Other possible biomarkers for the prediction of pathologic response after preoperative PBI that could be further investigated are magnetic resonance imaging, including functional imaging, and circulating tumor DNA.<sup>30-32</sup> With all these possible biomarkers, a large patient cohort will be necessary to differentiate between responders and nonresponders, as we expect only subtle differences in these low-risk patients.

## Conclusion

TILs could be determined in tumor tissue of low-risk patients before and after an ablative dose of preoperative PBI

using IHC staining. In this limited cohort, a statistically significant decrease in TILs was observed after irradiation. No differences in numbers of preirradiation TILs between responders and nonresponders were observed in this small group of low-risk patients with breast cancer.

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