Clinical Cancer Research

Association of CD4$^+$ Radiation-Induced Lymphocyte Apoptosis with Fibrosis and Telangiectasia after Radiotherapy in 272 Breast Cancer Patients with >10-Year Follow-up

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Abstract

Purpose: Radiation-induced lymphocyte apoptosis (RILA) has been suggested as a predictive assay for adverse late reactions after radiotherapy. Thus, low RILA values of T-lymphocyte subpopulations have been associated with increased risk for various endpoints at 2 to 3 years of follow-up. The purpose was to test if such associations persist for specific endpoints (subcutaneous fibrosis, telangiectasia) in breast cancer patients with at least 10 years of follow-up.

Experimental Design: Two hundred and seventy-two female patients who had received breast-conserving therapy within the German ISE study were included (median follow-up: 11.6 years). Radiotherapy-induced side effects were scored according to the Late Effects in Normal Tissues-Subjective, Objective, Management, and Analytic (LENT-SOMA) classification system. RILA in the CD4$^+$, CD8$^+$, and natural killer (NK) subpopulations from peripheral blood was analyzed by flow cytometry. Multivariate predictive modeling was performed including relevant clinical risk factors.

Results: Low CD4$^+$ RILA was associated with increased risk for both fibrosis ($P = 0.011$) and telangiectasia ($P < 0.001$). For fibrosis, the association was stronger outside the surgical area (Fib_{out}; $P = 0.004$) than within (Fib_{in}; $P = 0.17$). Predictive multivariate modeling including clinical risk factors yielded OR of 3.48 (95% confidence interval, 1.84–6.58) for any fibrosis and 8.60 (2.71–27.3) for telangiectasia. Addition of CD4$^+$ RILA to the clinical variables improved discrimination (c statistics) from 0.62 to 0.68 for any fibrosis, 0.62 to 0.66 for Fib_{in}, 0.61 to 0.69 for Fib_{out}, and from 0.65 to 0.76 for telangiectasia. CD8$^+$ and NK RILA were not significantly associated with radiotherapy-related late reactions.

Conclusions: The results provide first evidence that low CD4$^+$ RILA is associated with increased subcutaneous fibrosis and telangiectasia even after 10 years. This supports the potential usefulness for predicting individual clinical risk.

Introduction

Radiotherapy (RT), in combination with surgery, chemotherapy, and immunotherapy, is a fundamental part of modern multimodal antitumor therapy. More than 60% of all cancer patients treated with curative intent receive RT as part of their treatment (1). Earlier tumor detection and technological advances in treatment planning and delivery have resulted in an increasing number of long-term survivors of breast cancer (2, 3). Late normal-tissue reactions limit the dose of RT given to cancer patients and affect the quality of life of many patients. Thus identification of relatively radiosensitive or -resistant patients would enable improved personalized treatment, e.g., by modifying the dose, fractionation scheme, or offering an alternative treatment.

It has been estimated that up to 80% of the observed variation in risk of radiation-induced late reaction may be ascribed to factors associated with the individual patient (4). Over the last decades, various assays have been tested in search for predictive markers for individual risk that may be used to personalize RT for cancer patients. A number of these assays have tried to identify relevant genetic variants such as SNPs and, more recently, factors based on other "omics" including epigenetics, the transcriptome, proteome, kinome, and metabolome, thereby covering the entire spectrum from the genome to protein function (reviewed in ref. 5). Despite a large number of studies, only few significant genes have been identified, which is likely to be due to the involvement of multiple genes with small effect sizes in the development of RT-induced toxicities, thus requiring very large cohorts to achieve significance (6).

An alternative approach is to use functional assays which investigate the association between cellular endpoints

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Translational Relevance

Late normal-tissue reactions after radiotherapy affect the quality of life of many patients. The ability to identify patients with a predisposition for severe side effects before starting radiotherapy is a prerequisite for personalized treatment. The radiation-induced lymphocyte apoptosis (RILA) assay on specific lymphocyte populations from peripheral blood has been proposed as a simple functional assay to predict patients' individual clinical radiosensitivity. However, large studies on breast cancer patients with long-time follow-up are lacking. The present study shows for the first time a correlation between low $CD4^+$ RILA and increased risk of fibrosis and telangiectasia in breast cancer patients with minimum 10 years of follow-up. Predictive modeling suggests that this functional assay may help identify radiosensitive patients who can be offered less aggressive treatments, and more radioresistant patients who may be candidates for dose escalation or hypofractionated radiotherapy schedules.

(e.g., DNA damage and repair, clonogenic cell survival, or apoptosis) and the risk of developing clinical normal-tissue reactions (5). A promising functional assay is the radiation-induced lymphocyte apoptosis (RILA) assay on lymphocyte subpopulations from peripheral blood (PB) which can be performed with standard flow cytometry equipment (7). A prospective study of 399 patients with mixed malignancies showed an inverse correlation between RILA values for $CD4^+$ or $CD8^+$ T lymphocyte populations after *in vitro* irradiation with 8 Gy and the risk of any late toxicity scored by the Common Toxicity Criteria (CTC v2.0) 6 to 24 months after RT (median dose 66 Gy in 1.8-2.0 Gy/fx; ref. 8). The correlation with late reactions was further supported by a small study of 26 breast cancer patients (9), whereas two other studies ($n = 59$ and $n = 16$, respectively) failed to detect a significant correlation with breast appearance (10, 11). However, recently, a significant inverse correlation between RILA in $CD8^+$ T cells and radiation-induced fibrosis was confirmed in a study of 456 breast cancer patients with up to 36 months of follow-up (12). Furthermore, similar correlations for $CD8^+$ RILA have been found for late reactions in head and neck ($n = 79$) and cervical cancers ($n = 94$), as well as for genitourinary but not gastrointestinal late reaction in prostate patients ($n = 198$; refs. 13–15). Notably, most of the studies have used rather broad toxicity scores, often including several endpoints that may not be biologically related. Furthermore, although late reactions may continue to appear 5 to 10 years after RT, studies with long-term follow-up have not been published so far.

Because long-term follow-up data from prospective, clinical RILA studies may not be available soon, we validated the RILA assay in 272 PB samples from female early breast cancer patients who had been treated with breast conserving surgery (BCS) and RT and followed prospectively in the German ISE cohort study (16). The patients had a minimum follow-up of 10 years, and late reactions were scored according to the Late Effects in Normal Tissues-Subjective, Objective, Management and Analytic (LENT-SOMA) classification system (17) with well-defined criteria for the specific endpoints, subcutaneous fibrosis, and telangiectasia. Because the $CD8^+$ lymphocyte population may include $CD3^-/CD8^+$ natural killer (NK) cells, three-color FACS was used to distinguish RILA of the $CD4^+$, $CD8^+$, and $CD3^-/CD8^+$ populations.

Materials and Methods

Patients and data collection

Four hundred and seventy-eight female patients with histologically confirmed early breast cancer or *in situ* carcinoma from the German ISE cohort (16) were treated with BCS during 1998–2001. The patients were recruited from the Rhine-Neckar region in Germany (Women's Clinic Heidelberg, St. Vincentius Clinic Karlsruhe, City Hospital Karlsruhe, and Universitätsmedizin Mannheim). In brief, breast cancer patients were eligible if they were treated unilaterally with RT (but without chemotherapy) after BCS. Adjuvant RT was given to the whole breast (50.0 Gy in 2.0 Gy/fraction or 50.4 Gy in 1.8 Gy/fraction) followed by a tumor bed boost of 6 to 16 Gy (median: 10 Gy, 2.0 Gy/fx; $n = 224$), or 56.0 Gy given in 2.0 Gy fractions without a boost ($n = 28$). Because of the higher dose to the whole breast in patients without boost, the difference in total dose was modest and did not result in a significant difference in fibrosis ($P = 0.85$) nor telangiectasia ($P = 0.65$). The equivalent dose in 2 Gy fractions (EQD2; calculated using the linear-quadratic model with α/β ratios of 2 Gy for fibrosis and 3 Gy for telangiectasia; ref. 18) was 60 Gy (47.9 Gy–70.0 Gy) for fibrosis and 60 Gy (48.4 Gy–70.0 Gy) for telangiectasia. Among the 409 patients alive at 10 years of follow-up, a total of 272 patients agreed to participate in the present study. Twenty patients were excluded: 6 due to mastectomy, contralateral breast cancer, or additional RT, 2 due to receiving high-dose brachytherapy, and 12 patients due to technical failing of the RILA assay. Thus, a total of 252 patients with a median follow-up of 11.6 years (range, 10.3–12.8 years) were available for data analysis (Table 1; Fig. 1). Subcutaneous fibrosis and telangiectasia of the irradiated breast were assessed by a single-study physician using the LENT SOMA scale (17). Furthermore, the location of fibrosis within or outside the surgical area was scored. Maximum early toxicity was scored dichotomously (grade, 0–2b vs. 2c and higher) using a modified CTCAE v2.0 scale, as described previously (16). Information on demographic factors, medical history, and lifestyle factors was gathered by means of self-reported questionnaires. The study was approved by the local ethics committee and was conducted according to Declaration of Helsinki principles. Written-informed consent was obtained from each patient.

Sample collection, preparation, and flow cytometry

At the last follow-up after a minimum of 10 years, 5 mL of PB was collected from each patient in heparin-coated syringes (Sarstedt) and stored overnight at room temperature to eliminate the potential influence of differences in storage times for the samples taken during the day and delivered to the lab in the evening. The next morning, PB was diluted 1:10 in DMEM supplemented with 20% FBS (Biochrom) and irradiated with 8 Gy of 6 MV X-rays or sham irradiated for baseline values (0 Gy). After irradiation, samples were incubated for 48 hours in a humidified cell culture incubator at 37°C under 5% CO_2. After incubation, the cells were harvested and 2 mL used for each staining. Samples were stained with CD3/CD4-FITC, CD8-PE, and appropriate IgG controls for FITC and PE (all BD-Pharmingen) for 15 minutes at room temperature and then red blood cells were lysed and fixed (FACS lysing solution, BD), according to the

Table 1. Characteristics of the German ISE breast cancer patient cohort

	Apoptosis assay ($N = 252$)	No apoptosis assay ($N = 226$)			
		Refused	Deceased	Technical problems	Exclusions
	N (%)	*N* (%)	*N* (%)	*N* (%)	*N* (%)
All	252 (100.0)	133 (100.0)	72 (100.0)	12 (100.0)	9 (100.0)
Age in years at surgery					
<60 years	131 (52.0)	60 (45.1)	22 (30.6)	8 (66.7)	6 (66.7)
≥60 years	121 (48.0)	73 (54.9)	50 (69.4)	4 (33.3)	3 (33.3)
Hypertension at diagnosis					
No	177 (70.2)	78 (58.6)	44 (61.1)	7 (58.3)	6 (66.7)
Yes	72 (28.6)	48 (36.1)	27 (37.5)	5 (41.7)	1 (11.1)
BMI in kg/m^2 at diagnosis					
<25	128 (50.8)	66 (49.6)	31 (43.1)	5 (41.7)	6 (66.7)
≥25	121 (48.0)	62 (46.6)	40 (55.6)	7 (58.3)	1 (11.1)
Hormonal treatment					
No	41 (16.3)	16 (12.0)	6 (8.3)	4 (33.3)	3 (33.3)
Yes	205 (81.3)	113 (85.0)	64 (88.9)	8 (66.7)	6 (66.7)
Smoking status at 10-year FU					
Never	163 (64.7)	91 (68.4)	52 (72.2)	9 (75.0)	4 (44.4)
Ever	83 (32.9)	36 (27.1)	19 (26.4)	3 (25.0)	3 (33.3)
T classification of TNM					
0	1 (0.4)	—	—	—	—
1	180 (71.4)	93 (69.9)	36 (50.0)	6 (50.0)	5 (55.6)
2	46 (18.3)	29 (21.8)	32 (44.4)	4 (33.3)	2 (22.2)
4	1 (0.4)	—	—	—	—
In situ	23 (9.1)	10 (7.5)	3 (4.2)	2 (16.7)	2 (22.2)
N classification of TNM					
0	205 (81.3)	99 (74.4)	48 (66.7)	7 (58.3)	5 (55.6)
1	24 (9.5)	21 (15.8)	18 (25.0)	3 (25.0)	2 (22.2)
2	—	—	2 (2.8)	—	—
M classification of TNM					
0	171 (67.9)	78 (58.6)	45 (62.5)	7 (58.3)	6 (66.7)
1	1 (0.4)	1 (0.8)	—	—	—
Acute side effect[a]					
No	202 (80.2)	112 (84.2)	62 (86.1)	8 (66.7)	8 (88.9)
Yes	50 (19.8)	21 (15.8)	10 (13.9)	4 (33.3)	1 (11.1)
Breast fibrosis score at 10-year FU					
0	79 (31.3)	6 (4.5)	—	5 (41.7)	—
1	96 (38.1)	12 (9.0)	—	6 (50.0)	—
2	64 (25.4)	10 (7.5)	—	1 (8.3)	2 (22.2)
3	13 (5.2)	—	—	—	1 (11.1)
Telangiectasia at 10-year FU					
0	195 (77.4)	19 (14.3)	—	7 (58.3)	2 (22.2)
1	33 (13.1)	5 (3.8)	—	4 (33.3)	—
2	17 (6.7)	3 (2.3)	—	1 (8.3)	1 (11.1)
3	7 (2.8)	1 (0.8)	—	—	—

NOTE: The percentages may not add up to 100% due to patients with unknown parameter (<6% of patients, except for the TNM variables).
[a]Modified CTCAE v2 score with 2c (≥1 moist desquamation or interruption of treatment due to side effects) and higher versus 0 to 2b, maximum grade during treatment.

manufacturer's instructions. After washing with PBS, samples were resuspended and incubated for 10 minutes at room temperature in PBS supplemented with RNAse (100 μg/mL; Serva) and the DNA dye 7-Aminoactinomycin (7-AAD; 4 μg/mL; Thermo Fisher). After staining, cells were acquired on a FACSCanto II (BD) and analyzed using the FlowJo 7.6.4 software package (Tree Star). A live gate was applied during measurement to exclude granulocytes, as these are not of relevance for the assay and may be mistaken for $CD4^+$ or $CD8^+$ lymphocytes owing to their strong autofluorescence. A total of 20,000 live-gated events were acquired per sample. For each of the respective lymphocyte subpopulations (CD4, CD8, $CD3^-$/$CD8^+$), the 7-AAD^+ population was gated and plotted as 7-AAD versus FSC. 7-AAD^{dull}-FCS^{low} cells were gated and defined as apoptotic, as these have a less than 2n DNA content and a reduced cell size (apoptotic phenotype; procedure modified from Menz and colleagues; ref. 19). RILA was defined as the percent apoptosis after 8 Gy minus the percent apoptosis at 0 Gy.

In vitro irradiation

All *in vitro* irradiations were performed with 6 MV X-rays from a clinical linear accelerator (Versa HD, Elekta Synergy) at a dose rate of 6.67 Gy/min using a 40×40 cm^2 irradiation field. The samples were irradiated in 25 mL cell-culture flasks (Falcon, Corning B.V. Life Sciences) at 100 cm source-surface distance with 15 mm water-equivalent material for dose buildup and 8 cm for backscatter.

Data and statistical analysis

Patient samples were blinded to avoid bias and were unblinded for data analysis after completing the RILA measurements. Data are presented as median and range, unless otherwise noted. To determine the relationship between two parametric variables, univariate linear regression was performed. The nonparametric Wilcoxon/Kruskal–Wallis test was used for comparison of two groups and the Spearman rho test

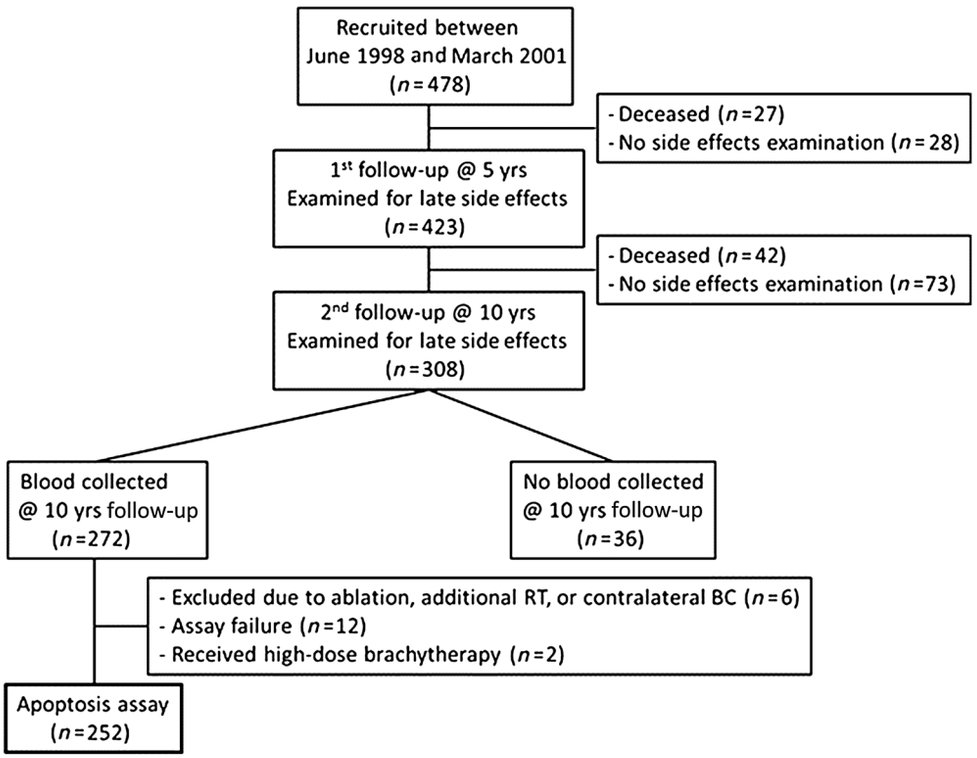

Figure 1.
Flow chart of the study population showing the development of the apoptosis assay cohort ($n = 252$) out of the initial ISE study cohort ($n = 478$).

for correlations with toxicity grade. ROC curves were created using RILA as a univariate random variable (classifier) and quantified by the AUC. For the determination of other predictive parameters [sensitivity, specificity, positive-predictive value (PPV), and negative-predictive value (NPV)], the RILA values were split into tertiles as proposed previously (8) and the middle and upper tertiles combined versus the lower tertile to obtain a binary variable for univariate analysis of the dichotomized toxicity (fibrosis/telangiectasia grade 2+3 vs. 0+1) similar to a recent study (12). It was verified that the proportions of patients with fibrosis in the middle and upper RILA tertiles were not significantly different in univariate analysis ($P = 0.08$, Fisher exact test), whereas the proportions with fibrosis were significantly larger in the lower tertile compared with the middle ($P < 0.0001$) and the upper tertiles ($P = 0.014$). Furthermore, multivariate analysis was performed adjusting for established and putative clinical covariables (12, 16). The final multivariate model included: Age at surgery (years, continuous), body mass index (BMI, in kg/m^2, continuous), hypertension (yes vs. no), smoking status at 10-year follow-up (ever vs. never), and the EQD2 (18). The model on fibrosis was additionally adjusted for hormonal treatment (yes vs. no; according to ref. 12). It was confirmed that the middle tertile was not associated with an increased risk (OR) when compared with the upper tertile as reference, in contrast with the lower tertile (Supplementary Table S1). Although a lower OR was observed for the middle tertile, it was only marginally significant [OR, 0.43 (0.17–0.97); $P = 0.04$] and may be coincidental because no mechanism is known that would predict this finding and the significance would be lost after correcting for multiple testing. Univariate statistical analyses were performed using JMP11 statistical software (SAS Institute). Graphs were plotted using either JMP11 or SigmaPlot 11.0 (Systat Software GmbH). Multivariable logistic regression analysis and bootstrapping analysis were performed using SAS 9.4 software.

As surgery may affect the development of fibrosis, and the influence of wound healing and scar formation can be assumed to be minimal outside the surgical area, we also assessed fibrosis according to location within the surgical area only (group 1) or outside (group 2) using multinomial logistic regression. Patients without subcutaneous fibrosis ≥grade 2 were used as controls (group 0). Adjustment variables were the same as in the binary logistic regression models with overall fibrosis. To assess robustness of the multivariate model, a bootstrapping analysis was performed based on 1,000 replications. The reported c statistic was corrected for optimism (18).

Results

RILA assay

In order to distinguish NK and different T-cell populations, triple-color FACS using 7-AAD for DNA staining was used instead of two-color FACS with propidium iodide (PI) as used in the original assay (8, 19). To verify that this did not influence the RILA values, pilot experiments with both fluorescent dyes were performed. A very strong correlation ($r^2 = 0.953$; $P < 0.001$) between the PI and 7-AAD RILA was observed for all three T-cell

populations (Supplementary Fig. S1) confirming the validity of replacing PI with 7-AAD.

T-cell populations and RILA

The distribution of RILA values for each lymphocyte population ($CD4^+$, $CD8^+$, and $CD3^-/CD8^+$) showed the largest range and highest median rate of radiation-induced apoptosis for NK cells ($CD3^-/8^+$), followed by $CD8^+$ and $CD4^+$ T cells (Fig. 2A; Supplementary Table S2). However, NK cells were much less abundant and more radiosensitive than $CD8^+$ and $CD4^+$. Thus based on 20,000 events, the median cell numbers were 419 (0 Gy) and 38 (8 Gy) for $CD3^-/CD8^+$, 2,441 (0 Gy) and 1,036 (8 Gy) for $CD8^+$, and 4,222 (0 Gy) and 3,373 (8 Gy) for $CD4^+$. RILA for the $CD4^+$ and $CD8^+$ lymphocyte populations revealed a significant correlation ($r^2 = 0.603$, $P < 0.001$; Supplementary Fig. S2), whereas only weak though significant correlations were found between $CD4^+$ or $CD8^+$ and the $CD3^-/8^+$ RILA values ($r^2 = 0.082$, $P < 0.001$ and $r^2 = 0.116$, $P < 0.001$, respectively). Notably, $CD8^+$ RILA values appear to be "capped" at 60% to 65%, whereas this is not the case for $CD4^+$ RILA. Thus, CD4 provides better discrimination than CD8 at high RILA values, whereas the variation between the two values dominates at low RILA values. As RILA was previously found to decrease with age (20), this was tested in the present cohort. No significant correlation with patient age was found in the range of 50.6 to 91.1 years (median, 70.7 years) for $CD4^+$ [0.026% per year; 95% confidence interval (CI), −0.102%, 0.154%; $r^2 < 0.001$, $P = 0.694$; Fig. 2B] and $CD3^-/CD8^+$ RILA (0.031% per year, 95% CI, −0.319%, 0.382%; $r^2 < 0.001$, $P = 0.860$; Fig. 2C). By contrast, a very weak but significant increase of $CD8^+$ RILA with age (0.267% per year; 95% CI, 0.034%–0.499%; $r^2 = 0.016$, $P = 0.025$) was observed (Fig. 2D). Thus, a decrease in RILA with increasing age was not observed in the age range (50.6–91.1 years) of this cohort.

Radiation-induced normal-tissue toxicity and RILA

Ten years after RT, 30.6% of the patients had developed subcutaneous fibrosis grade ≥2 and 9.5% telangiectasia grade ≥2, with 6.7% developing both (Table 1). Radiation-induced maximum early effects (grade ≥ 2c) were observed in 19.8% of the patients. RILA values were significantly lower in patients with RT-induced fibrosis compared with unaffected patients for $CD4^+$ cells ($P = 0.011$) but not for $CD8^+$ or $CD3^-/CD8^+$ cells (Fig. 3A).

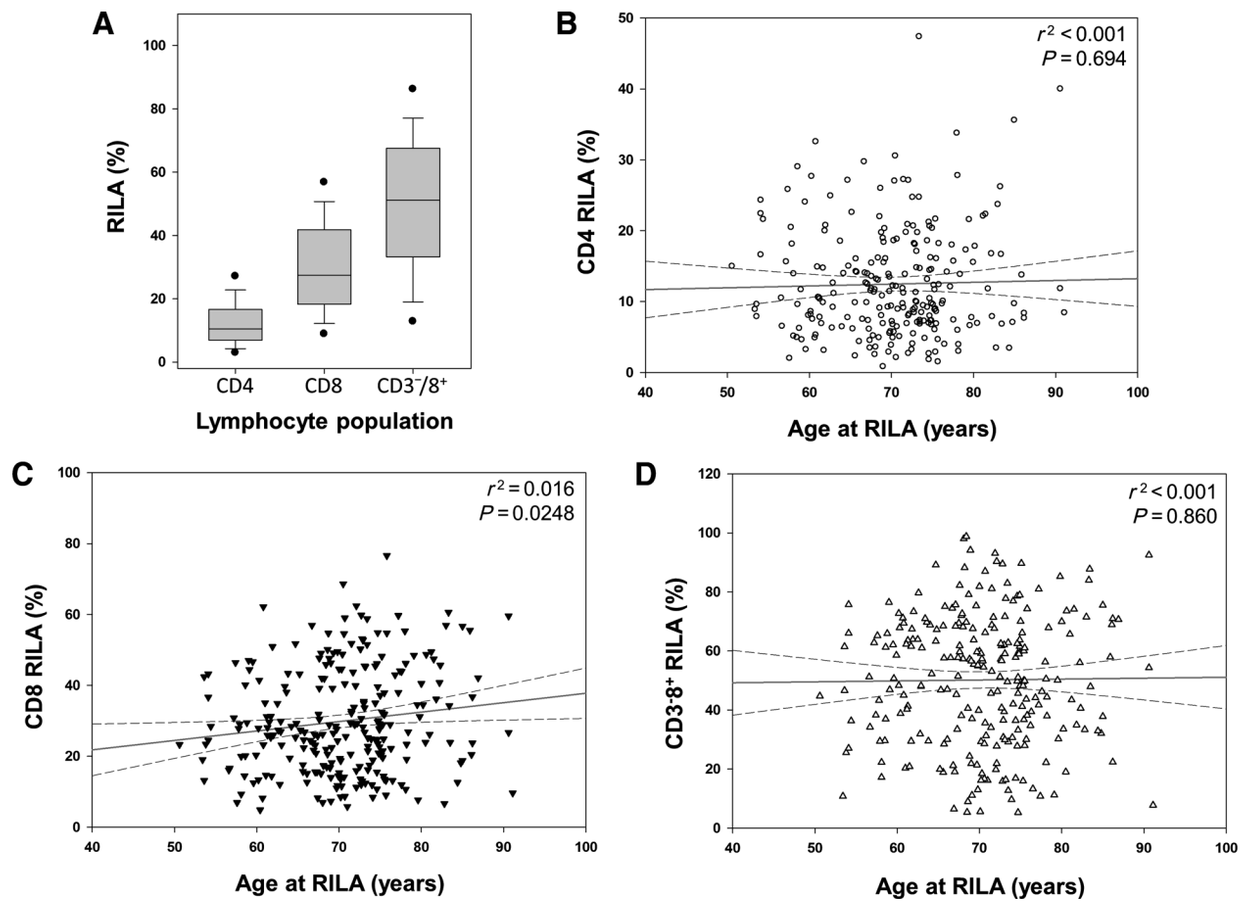

Figure 2.

Characteristics of RILA. Box plot showing the RILA values in percent and their distribution for each of the three lymphocyte populations ($CD4^+$, $CD8^+$, and $CD3^-/CD8^+$; **A**). Whiskers depict the 10th and 90th percentiles, and filled circles depict the 5th and 95th percentiles. The effect of patient's age on $CD4^+$ (**B**), $CD3^-/CD8^+$ (**C**), and $CD8^+$ (**D**) RILA. No significant correlation was detected between age of the patient and $CD4^+$ or $CD3^-/CD8^+$ RILA, whereas for $CD8^+$ RILA, a weak positive correlation with age of the patient was observed. The dotted black lines flanking the regression line (gray) indicate the 95% CIs.

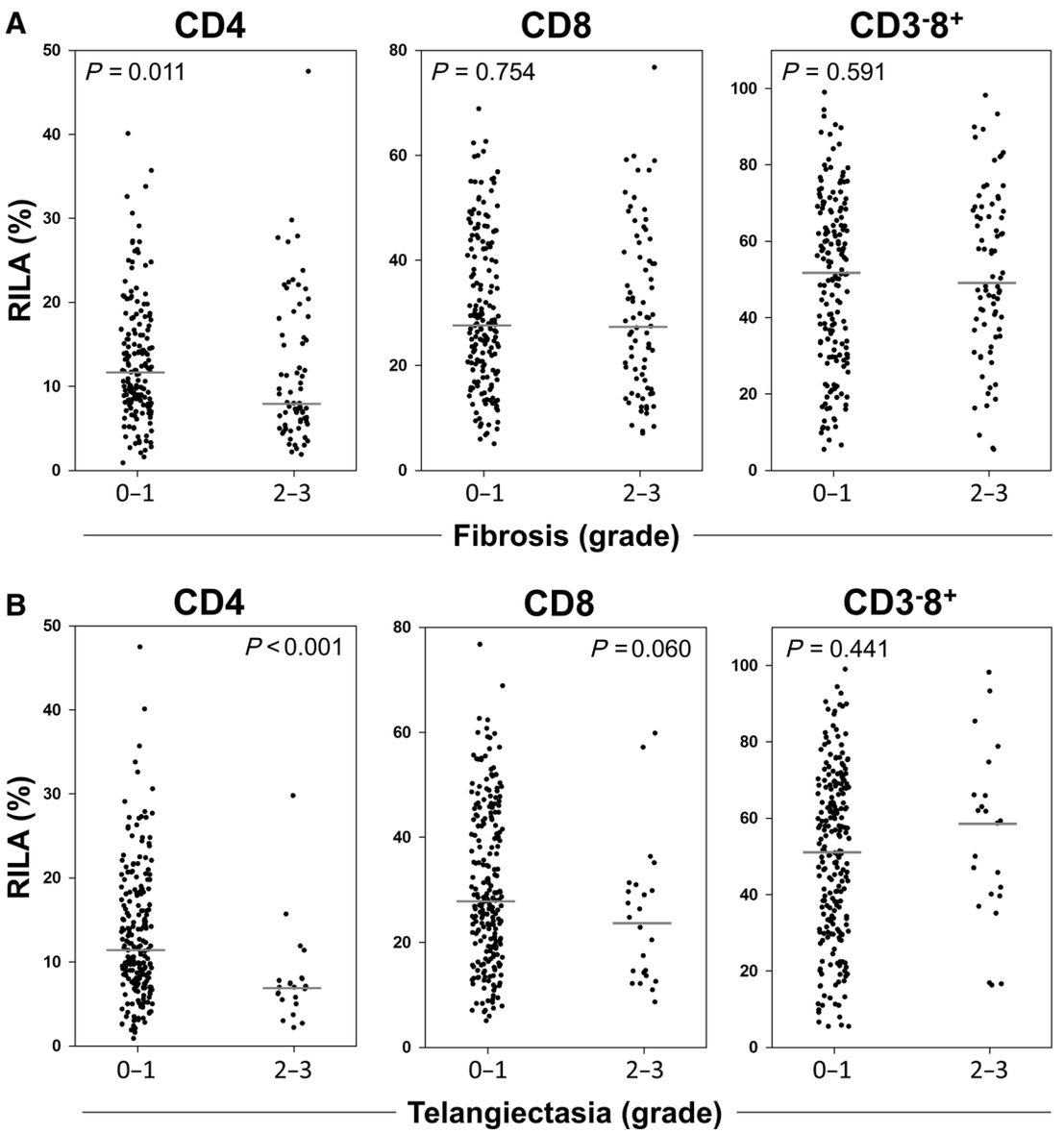

Figure 3.

Scatter plot of RILA values for patients with mild or no late reaction (grade 0–1) vs. moderate or severe reaction (grade 2–3). Data are shown for each lymphocyte population ($CD4^+$, $CD8^+$, and $CD3^-/CD8^+$) for fibrosis (**A**) or telangiectasia (**B**). Significant associations were found for $CD4^+$ and fibrosis ($P = 0.011$) or telangiectasia ($P < 0.001$). A trend was observed for $CD8^+$ and telangiectasia ($p_{tel.} = 0.060$), whereas all other comparisons were nonsignificant ($CD8^+$ $p_{fibr.} = 0.754$; $CD3^-/CD8^+$ $p_{fibr.} = 0.5919$, $p_{tel.} = 0.441$). The gray horizontal line represents the median value for each group.

The difference in $CD4^+$ RILA was even more pronounced for telangiectasia ($P < 0.001$) and showed a similar trend for $CD8^+$ RILA ($P = 0.060$; Fig. 3B). The distribution of $CD4^+$ RILA values seemed to be more skewed toward lower values, especially for telangiectasia. No association between basal apoptosis for any of the lymphocyte populations and the onset of fibrosis ($p_{CD4} = 0.84$; $p_{CD8} = 0.67$; $p_{NK} = 0.78$) or telangiectasia ($p_{CD4} = 0.56$; $p_{CD8} = 0.79$; $p_{NK} = 0.61$) was observed.

The correlation between the grade of late reactions and RILA values corroborated the association between the $CD4^+$ RILA and the risk of developing fibrosis or telangiectasia (rho_{fibr}: −0.171, $P = 0.008$; rho_{tel}: −0.205, $P = 0.002$; Supplementary Fig. S3A). For $CD8^+$ lymphocytes, no significant correlation of RILA was detected with grade of fibrosis (rho: −0.006; $P = 0.920$) nor telangiectasia (rho: −0.122; $P = 0.053$; Supplementary Fig. S3B). In addition, no significant association between RILA values and maximum early RT-induced toxicity (2c and higher vs. grade 0–2b) was observed for any of the lymphocyte populations ($P > 0.6$; Supplementary Fig. S4).

In multivariate analysis of RILA, including relevant clinical risk factors, the lower $CD4^+$ RILA tertile was significantly associated with an increased OR for both fibrosis (OR, 3.48; 95% CI,

Table 2A. Multivariate association of $CD4^+$ and $CD8^+$ RILA with breast fibrosis and telangiectasia at long-term follow-up

	Breast fibrosis		Telangiectasia	
	P	**OR (95% CI)**	***P***	**OR (95% CI)**
$CD4^+$ RILA	0.0001	3.48 (1.84–6.58)	0.0002	8.60 (2.71–27.3)
$CD8^+$ RILA	0.29	1.39 (0.75–2.57)	0.136	1.99 (0.81–4.92)

Table 2B. Multinomial logistic regression analysis according to location of breast fibrosis: within the surgical area only ($n = 54$) or outside the surgical area ($n = 23$); patients without fibrosis were used as control group in both cases ($n = 175$)

	Fibrosis within the surgical area only		Fibrosis outside the surgical area	
	P	**OR (95% CI)**	***P***	**OR (95% CI)**
$CD4^+$ RILA	0.0022	3.08 (1.50–6.34)	0.0040	4.33 (1.60–11.7)
$CD8^+$ RILA	0.39	1.35 (0.68–2.69)	0.41	1.51 (0.57–3.99)

NOTE: Outcome: dichotomized as score 2 and higher vs. 0 and 1. RILA values dichotomized as low versus middle and upper tertiles. Variables of the multivariate model: Age at surgery (years), BMI (kg/m^2), hypertension (yes vs. no), smoking status at 10-year follow-up (ever vs. never smokers), and delivered radiotherapy dose (EQD2). The model on fibrosis was additionally adjusted for hormonal treatment (yes vs. no).

1.84–6.58) and telangiectasia (OR, 8.60; 95% CI, 2.71–27.3, Table 2A) compared with the middle/upper tertiles, whereas no association was observed for $CD8^+$ RILA, neither with fibrosis nor with telangiectasia.

A subanalysis according to location of fibrosis with respect to the surgical area was performed, based on the assumption that fibrosis outside the surgical area was more likely to be related to RT with minimal influence of wound healing and scar formation after surgery (Supplementary Table S3). Using the nonparametric Wilcoxon/Kruskal–Wallis test, the distribution of $CD4^+$ RILA values was not correlated with fibrosis limited to the surgical area (group 1, $P = 0.17$, $n = 54$) but significantly associated with fibrosis outside the surgical area (group 2, $P = 0.004$, $n = 23$; Supplementary Fig. S5). No significant differences were observed for $CD8^+$ RILA ($p_{\text{within only}} = 0.938$; $p_{\text{outside}} = 0.478$). In multivariate logistic regression analysis, fibrosis outside the surgical area (Table 2B, $n = 23$) revealed higher ORs (4.33, 95% CI, 1.60–11.7) for low $CD4^+$ RILA compared with overall fibrosis (Table 2A, OR = 3.48) or fibrosis limited to the surgical area (Table 2B, OR = 3.08). However, the difference in OR between fibrosis outside versus within the surgical area was not statistically significant ($P = 0.5$).

Predictive univariate ROC analysis yielded an AUC of 0.604 for predicting fibrosis from $CD4^+$ RILA and 0.736 for telangiectasia, whereas the values for $CD8^+$ were lower (0.511 and 0.617, respectively; Table 3). Notably, prediction of fibrosis outside the surgical area from $CD4^+$ RILA was markedly better with AUC increased to 0.687. Comparing the lower tertile versus the upper two tertiles yielded 77% sensitivity and 71% specificity for $CD4^+$ RILA prediction of telangiectasia $\geq$ grade 2 in the present cohort (Table 3). However, the NPV (97%) was much higher than the PPV (22%). Values for predictive modeling of fibrosis and for $CD8^+$ RILA as a potential predictor are shown in Table 3. The NPV was increased (79%→93%) at the expense of the PPV (48%→22%) for $CD4^+$ RILA and fibrosis outside the surgical area compared with any fibrosis (Table 3). With respect to the severity of late reaction, for $CD4^+$ RILA, 53% (38/72) of patients with fibrosis (grade $\geq$ 2) and 69% (9/13) of the grade 3 cases were in the lower tertile of the samples. Stratifying by the location of $\geq$ grade 2 subcutaneous fibrosis yielded 51% (25/49) of all patients with fibrosis within and 61% (14/23) of all patients with fibrosis outside the surgical area in the lower tertile. For grade 3, this was observed in 60% (3/5) of the patients with fibrosis within and 100% (4/4) of the patients with fibrosis outside the surgical area. For telangiectasia $\geq$grade 2, the fractions of patients in the lower tertile were 77% (17/22) and 100% (7/7) for grade 3.

To estimate the predictive value of RILA in the presence of clinical risk factors, bootstrapping was performed. The results showed a moderate predictive value of $CD4^+$ based on the corrected C statistics, which is equivalent to the AUC in an ROC analysis (Table 3), including $CD4^+$ in the model improved discrimination (c statistics) from 0.62 to 0.68 for any breast fibrosis, from 0.62 to 0.66 for fibrosis within the surgical area only, from 0.61 to 0.69 for fibrosis outside the surgical area, and from 0.65 to 0.76 for telangiectasia. Addition of $CD8^+$ RILA to the model did not improve prediction of fibrosis nor telangiectasia when adjusted for clinical risk factors (Table 3), implying that $CD4^+$ RILA may be a more robust predictor of radiation-induced late reaction at 10 years of follow-up than $CD8^+$ RILA.

Discussion

In the present study, we tested the association between RILA and RT-induced late toxicities in 272 early breast cancer patients with long-term follow-up after RT (range, 10.3–12.8 years) in different lymphocyte subpopulations. RILA in $CD4^+$ (T-helper) cells was strongly associated with telangiectasia after RT and, to a lesser extent, with subcutaneous fibrosis. The association was somewhat stronger for fibrosis outside the surgical area where the confounding influence of wound healing and scar formation was likely to be small. By contrast, RILA in the cytotoxic T-cell population ($CD8^+$) did not significantly correlate with fibrosis or telangiectasia. In addition, there was no association of RILA for the highly radiosensitive NK-cell ($CD3^-/8^+$) population with late toxicity or between RILA for any of the three lymphocyte populations and early toxicity after RT. Multivariate analysis for $CD4^+$ RILA accounting for clinical risk factors showed significantly increased ORs for both fibrosis and telangiectasia when comparing the lower tertile with the upper two RILA tertiles.

To our knowledge, this is the first study showing a strong association of T-cell RILA with telangiectasia as well as an association of $CD4^+$ RILA with fibrosis in a large breast cancer patient cohort. The observation that all patients with telangiectasia or severe fibrosis outside the surgical area had $CD4^+$ RILA values in the lower tertile (<7.9%) corroborates the association. Barber and colleagues (21) investigated the rate of apoptosis (using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay) in total PB lymphocytes from 31 breast cancer patients, but did not observe any correlation between a reduction in apoptosis and breast fibrosis or telangiectasia 8 to 13 years after RT. In a different study, a correlation with reduced late reaction (mainly breast shrinkage and subcutaneous fibrosis) at long follow-up (mean 16.4 years) was found in a small cohort ($n = 26$) for a predictor combining high radiation-induced apoptosis and low double-strand break induction (9). It should be noted that in these studies, no labeling of lymphocyte subpopulations was performed, and apoptosis was measured by different methods which may have contributed to the conflicting results. Two further studies on breast cancer patients did not find a significant correlation with

Table 3. Uni- and multivariate predictive modeling of RT-related late reactions for the RILA assay; the AUC was determined from ROC analysis based on RILA values from each individual sample; for determination of the other predictive parameters, the RILA data were split into equal tertiles[a] and the lower tertile tested against the middle plus upper tertiles; discriminative parameters (univariate) were determined for each group in addition to bootstrapping (multivariate); the latter was performed to estimate the predictive value of RILA in the presence of clinical risk factors, which was adjusted for optimism (corrected C statistics)

	Univariate					Multivariate	
	AUC_{ROC}	Sensitivity	Specificity	PPV	NPV	C stats[b] RILA	C stats[b] without RILA
Any breast fibrosis							
$CD4^+$ RILA	0.604	52.8%	75.2%	48.1%	78.5%	0.675	0.623
$CD8^+$ RILA	0.512	36.4%	68.0%	33.1%	70.8%	0.622	
Fibrosis (within only)[c]							
$CD4^+$ RILA	0.564	51.0%	72.1%	35.2%	83.2%	0.661	0.621
$CD8^+$ RILA	0.503	35.2%	66.9%	24.7%	77.0%	0.616	
Fibrosis (outside)[d]							
$CD4^+$ RILA	0.687	60.9%	70.3%	22.2%	92.8%	0.694	0.605
$CD8^+$ RILA	0.546	39.1%	67.4%	13.6%	89.4%	0.604	
Telangiectasia							
$CD4^+$ RILA	0.736	77.3%	71.2%	21.5%	96.8%	0.758	0.651
$CD8^+$ RILA	0.617	45.8%	68.0%	13.1%	92.3%	0.658	

NOTE: Adjustment variables: Age at surgery (years), BMI (kg/m^2), hypertension (yes, no), smoking status at 10-year follow-up (ever vs. never smokers), and delivered radiotherapy dose (EQD2). The model on fibrosis was additionally adjusted for hormonal treatment (yes vs. no).

[a]Definition of tertiles: For any breast fibrosis and telangiectasia: $RILA_{CD4}$: < 7.9%/7.9%–14.1%/>14.1%; $RILA_{CD8}$: <22.0%/22.0%–34.9%/>34.9%. For fibrosis outside as well as fibrosis exclusively within the surgical area: $RILA_{CD4}$: <8.6%/8.6%–13.9%/>13.9%; $RILA_{CD8}$: <22.4%/22.4%–34.5%/>34.5%.

[b]Corrected C statistics.

[c]Fibrosis occurring exclusively within the surgical area.

[d]Fibrosis occurring outside the surgical area.

breast appearance after RT at median follow-up of 12.5 years ($n = 59$) and 5 years ($n = 16$), respectively (10, 11). However, the larger of these studies used lymphocytes which had been stored frozen as viable cells (10, 11), whereas all other studies have used fresh lymphocytes.

A prospective study by Ozsahin and colleagues found significant inverse correlations of $CD4^+$ and $CD8^+$ RILA with any moderate to severe late toxicity scored by CTCAE v2.0 in a large diverse patient cohort including 149 breast cancer patients (8). Based on a better prediction by $CD8^+$ compared with $CD4^+$ RILA in this study, Azria and colleagues recently conducted a prospective multicenter study on the association of $CD8^+$ RILA with subcutaneous fibrosis (but not telangiectasia) in a cohort of 456 breast cancer patients (12). RILA was significantly lower (median 10.9%) for patients with moderate to severe breast fibrosis (CTCAE 3.0) within 3 years than for patients without reactions (median 15.6%; $P < 0.001$), thereby confirming the previous association of $CD8^+$ RILA with broad late end points (8) for a specific end point in breast cancer patients. ROC analysis yielded an AUC of 0.62 (95% CI, 0.54–0.70). The present study, unexpectedly, found no correlation between $CD8^+$ RILA and any fibrosis (AUC = 0.51), whereas univariate predictive modeling for $CD4^+$ RILA yielded an AUC of 0.60. This AUC value, as well as the predictive parameter based on $CD4^+$ RILA lower versus middle and upper tertiles, was comparable with the corresponding values for $CD8^+$ in the study by Azria and colleagues (12), with the exception of the PPV, which was higher in our study (NPV was not shown in ref. 12). In the latter article, predictive parameters were also determined for the lower plus middle versus the upper CD8 RILA tertiles. Performing this analysis on our RILA data (see Supplementary Table S4) revealed a similar picture for both $CD4^+$ and $CD8^+$ RILA: whereas the sensitivity was slightly higher in the previous study (80% vs. 69% and 66.2%, respectively), the specificity was identical (34% vs. 35% and 33.1, respectively) and the NPV was higher (91% vs. 72% and 69%, respectively; PPV was not shown in ref. 12). Interestingly, confining the location of the fibrosis to outside the surgical area strengthened the association with $CD4^+$ RILA (Supplementary Fig. S5) and adding $CD4^+$ RILA improved the AUC to 0.687 (compared with 0.604 of any fibrosis; Table 3) despite the lower number of cases. The results from the multivariate analysis showed the largest improvement in c statistics when $CD4^+$ RILA was included in the model for fibrosis outside (from 0.605 to 0.694) compared with any fibrosis (from 0.623 to 0.675) or fibrosis within the surgical scar area (from 0.621 to 0.661; Table 3). This suggests that wound healing is a confounding factor for fibrosis and that the risk of developing fibrosis within the scar area is determined mainly by clinical factors.

It is not clear why $CD4^+$ RILA correlated better with normal-tissue reaction than $CD8^+$ RILA in the present study with long follow-up. Although a correlation between $CD4^+$ and $CD8^+$ was observed, this was not strictly linear showing large relative variation at low values with decreasing correlation at high values. $CD8^+$ RILA values in the present study were approximately 10 percent points higher (lower tertile < 22.0%, middle 22.0%–34.9%, upper $\geq$ 34.9%) than in the study by Azria and colleagues (<12%, 12%–24%, and $\geq$24%; ref. 12) but appeared to be "capped" at 60% to 65%. The different values for separating the tertiles in different studies may be related to minor differences in sample processing and technical aspects (RILA protocol and timing, serum used in the medium, gating strategy, patient malignancy, etc.). These factors are currently being investigated as part of an international multicenter study funded by the FP7 program (REQUITE; https://requite.eu/). The age of the patients at the time of surgery was similar in the present (median 59 years; range 39–79) and in the other study (median 56 years; range 29–88), but the present blood samples were taken 10 to 13 years after treatment. Although $CD8^+$ RILA increased with age, the rate of change was too small (0.27% per year; upper 95% confidence limit: 0.50% per year) to explain the higher absolute values compared with the other study (12). Furthermore, $CD8^+$ and $CD4^+$ RILA values have previously been reported to decrease rather than increase with age (20), and RILA

values similar to the $CD8^+$ RILA in the present study have been published for unlabeled lymphocytes in a prospective study of breast cancer patients (9) with similar age distribution as in the study by Azria and colleagues (12). In addition, the present $CD8^+$ RILA values still showed a wide range from 5% to 70%–75%. Therefore, a systematic change or loss of variation in $CD8^+$ RILA with age does not explain the lack of association between late reaction and $CD8^+$ RILA. Although differences in the FACS analysis may contribute to the difference in absolute values, it cannot be excluded that $CD8^+$ RILA may change over time due to factors not related with the treatment and that $CD4^+$ RILA may be more robust than $CD8^+$ RILA.

Late reaction may continue to develop for several years after RT (22, 23). In the present study, 30.6% of the patients had developed ≥grade 2 subcutaneous fibrosis at 11.6-year median follow-up (range 10.3–12.7), whereas in the study by Azria and colleagues (12), 14.7% (64 of 434 patients with 3-year follow-up) had developed breast fibrosis up to 3 years (peak toxicity scored between 12 weeks to 36 months after RT). Although differences in treatments and clinical scoring (e.g., prevalence of tumor-bed boost; dose inhomogeneity; LENT-SOMA vs. CTCAE v3.0; different physicians) may play a role, the increased incidence at long-term follow-up is likely to be predominantly due to the continued risk of fibrosis (22, 23). If $CD8^+$ RILA was predictive for breast fibrosis developing up to 3 years but not events developing later, this might lead to loss of the correlation with $CD8^+$ RILA at long follow-up. A prospective study on 198 prostate cancer patients finding an association between $CD4^+$ but not $CD8^+$ RILA and genitourinary toxicity at 5.3-year median follow-up (15) might support this interpretation. A recent study on a breast cancer cohort ($n = 105$) with 6 years of median follow-up recorded only 5 cases of fibrosis as no boost was given (24, 25). Thus, although an association with $CD8^+$ RILA was reported, the published data, and the difference in RT treatment, did not allow a more detailed comparison of RILA distributions.

An intriguing observation in the present study is that telangiectasia correlated more strongly with the RILA assay than fibrosis, suggesting that RILA may be a better predictor for late vascular effects than for fibrosis. This might potentially help explain the loss of correlation with $CD8^+$ RILA with increasing follow-up time. Thus, if fibrotic events scored at early time points have a larger vascular component (e.g., edema) than at later time points, and if early vascular effects correlate with low $CD8^+$ RILA, this could provide a mechanism for a change in predictive value of $CD8^+$ as breast edema decreases and more breast fibrosis develops with time. To test this hypothesis, prospective studies with long-term follow-up, scoring both endpoints at regular intervals, will be required.

The association between low $CD8^+$ RILA and increased risk of late reaction has been proposed to be caused by a slow apoptotic response and enhanced production of cytokines that attract inflammatory cells into the tissue (8, 26). We speculate that a low propensity for $CD4^+$ apoptosis may favor polarization of Th cells toward an anti-inflammatory and fibrogenic response (Th_2 secreting IL4, IL5, IL13; regulatory T cells, T_{reg}, secreting IL10, TGFβ1). Thus, increased $CD4^+$ apoptosis after RT might result in a less anti-inflammatory and fibrogenic microenvironment conferring resistance in a subgroup of patients consistent with the hypothesis of multiple subgroups characterized by different mechanisms of late reaction (5, 27). Indeed, the two mechanisms are not mutually exclusive because the recovery of normal tissue from radiation injury resembles the complex dynamics of wound-healing processes starting with an inflammatory response followed by an anti-inflammatory phase and later tissue remodeling. During the final preparation of this article, Florian Wirsdörfer and Verena Jendrossek [Institute of Cell Biology (Cancer Research), University Hospital Essen, 45122, Essen, Germany] reported that in a murine model of radiation-induced pneumopathy, $CD4^+$ T lymphocytes are altered and contribute to profibrotic signaling and lung fibrosis [Oral presentation and personal communication at the Annual Conference of the German Association of Radiation Biology (Gesellschaft für Biologische Strahlenforschung) 2018, Frankfurt am Main, Germany]. These results corroborate our findings that low $CD4^+$ RILA values are associated with enhanced risk for RT-related adverse late effects of the skin and support the hypothesis that a low propensity for apoptosis of $CD4^+$ cells contributes to a fibrogenic response. An improved understanding of the role of immune cells and cytokines is likely to open new strategies for mitigating fibrosis (28) and possibly other adverse effects.

Although NK-cell RILA was included in an early study to determine the most suitable lymphocyte population for the RILA assay (7), the present study is the first to investigate its association with RT-induced toxicity in breast cancer patients. However, irradiation with 8 Gy almost completely eradicated NK cells making this population unsuitable for prediction at this dose level. This is consistent with a study on cervical cancer patients (13) but contrasts with a study on RT for prostate cancer (29). Both studies suggested that B cells might potentially be relevant. Thus, different lymphocyte populations, including NK cells at different dose levels, should be included in future studies and analyzed in relation to specific late endpoints.

The main limitation of the present study is that it is retrospective, although patients were recruited from the prospective ISE cohort. Thus, it is not possible to determine the true predictive values of the RILA assay. In particular, it is not possible to clarify if $CD8^+$ RILA would have been predictive for fibrosis scored at earlier follow-up. On the other hand, the RILA assay is expected to measure constitutional apoptotic propensity, and no prospective studies with similar long-term follow-up have been performed so far. All patients were followed for more than 10 years irrespective of when the adverse event occurred, and the rate of late reaction was twice that of studies limited to 2 to 3 years of follow-up. Nevertheless, the present study supports the previous findings that RILA values determined on patients' PB lymphocytes are associated with the individual risk of developing late reaction after RT. However, it raises a number of important questions: (1) Do the predictive values of $CD4^+$ and $CD8^+$ RILA change with follow-up time? (2) Do any such changes depend on the specific late endpoint? (3) Is the predictive value related to a role of the immune system in late reaction? And, if so, (4) Can late reaction be mitigated by cytokines or antibodies against specific cell types during or after RT? Answering these questions will require further prospective and mechanistic studies. Although some will be addressed in the ongoing REQUITE study, the results and questions from the present study emphasize the need for very long follow-up in prospective studies.

In conclusion, a significant association between RILA of $CD4^+$ T-cell populations and two specific RT-related late reactions (breast fibrosis and telangiectasia) in breast cancer patients after 10-year follow-up was shown for the first time. The association with telangiectasia was novel and was stronger than for fibrosis. For fibrosis, the association tended to be stronger outside the

surgical area than within. Predictive modeling supported the use of the RILA functional assay for personalized RT and suggested that $CD4^+$ RILA may be a more robust predictor over time than $CD8^+$ RILA. Finally, the present study raises important questions regarding the relevant lymphocyte population and mechanistic aspects which should be addressed in future prospective studies.

Disclosure of Potential Conflicts of Interest

E. Sperk reports receiving speakers bureau honoraria and commercial research support from Zeiss Meditec. F.A. Giordano reports receiving speakers bureau honoraria from Carl Zeiss Meditec AG, MSD Sharp & Dohme, NOXXON Pharma AG, and Roche Pharma AG, is a consultant/advisory board member for Carl Zeiss Meditec AG, Guebet SA, and NOXXON Pharma AG, and reports receiving commercial research grants from Carl Zeiss Meditec, ELEKTA AB, Guerbet SA, humediQ, and NOXXON Pharma AG. No potential conflicts of interest were disclosed by the other authors.

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